Quantitative Proteomics Reveals that Plasma Membrane Microdomains from Poplar Cell Suspension Cultures are Enriched in Markers of Signal Transduction, Molecular Transport and Callose Biosynthesis

Vaibhav Srivastava#, Erik Malm#, Gustav Sundqvist, Vincent Bulone*

From the Division of Glycoscience, School of Biotechnology, Royal Institute of Technology (KTH), AlbaNova University Centre, 106 91 Stockholm, Sweden

#These authors contributed equally to the work presented.

*To whom correspondence should be addressed: Vincent Bulone, Division of Glycoscience, School of Biotechnology, Royal Institute of Technology (KTH), AlbaNova University Centre, 106 91 Stockholm, Sweden. Tel.: +46 8 5537 8841; Fax: +46 8 5537 8468; E-mail: bulone@kth.se

Running title: The Proteome of Detergent-Resistant Microdomains from Poplar
The Proteome of Detergent-Resistant Microdomains from Poplar

ABBREVIATIONS
DGK, diacylglycerol kinase
DRM, detergent-resistant microdomains
GPI, glycosylphosphatidylinositol
GSL, glucan synthase-like
iTRAQ, isobaric tags for relative and absolute quantitation
PA, phosphatidic acid
PLD, phospholipase D
PM, plasma membrane
PMF, plasma membrane fraction
SCAMPS, secretory carrier membrane proteins
SCX, strong cation exchange chromatography
SNARE, SNAP receptor
TE, Triton extract
TEAB, triethylammonium bicarbonate
TMD, transmembrane domains
SUMMARY
The plasma membrane (PM) is a highly dynamic interface that contains detergent-resistant microdomains (DRM). The aim of this work was to determine the main functions of such microdomains in poplar through a proteomic analysis using gel-based and solution (iTRAQ) approaches. Compared to PM, a total of 80 proteins from a limited number of functional classes were found to be significantly enriched in DRM. The enriched proteins are markers of signal transduction, molecular transport at the PM or cell wall biosynthesis. Their intrinsic properties are presented and discussed together with the biological significance of their enrichment in DRM. Of particular importance is the significant and specific enrichment of several callose [(1→3)-β-glucan] synthase isoforms, whose catalytic activity represents a final response to stress, leading to the deposition of callose plugs at the surface of the PM. An integrated functional model that connects all DRM-enriched proteins identified is proposed. This report is the only quantitative analysis available to date of the protein composition of membrane microdomains from a tree species.
INTRODUCTION

The plasma membrane (PM) is considered as one of the most interactive and dynamic supramolecular structures of the cell (1, 2). It forms a physical interface between the cytoplasm and the extracellular environment and is involved in many biological processes such as metabolite and ion transport, gaseous exchanges, endocytosis, cell differentiation and proliferation, defense against pathogens, etc (3). Various combinations of biochemical and analytical approaches have been used to characterize the PM proteome in different organisms, e.g. yeast, plants and animals (4-8). Typically, PM proteins are either embedded in the phospholipid bilayer through transmembrane helices or less tightly bound to the membrane through reversible or irreversible surface interactions. In Eukaryotic cells, some PM proteins are enriched in lateral lipid patches that form microdomains within the membrane (9, 10). These microdomains are considered to act as functional units that support and regulate specific biological processes associated to the PM (9, 10). Often referred to as “membrane (lipid) rafts” in animals and other organisms, they are typically described as being enriched in sphingolipids, sterols and phospholipids that contain essentially saturated fatty acids (9, 10, 11). Early work on PM microdomains has suggested that their specific lipid composition confers resistance to certain concentrations of non-ionic detergents, such as Triton X-100 and NP-40 (10, 11). Although this property has been exploited to isolate experimentally so-called detergent-resistant microdomains (DRM), the relationship between DRM and membrane rafts remains controversial (12). Indeed, the relation between the two is much debated, essentially because the use of Triton X-100 at 4°C to prepare DRM has been proposed to potentially induce the artificial formation of detergent-resistant structures whose composition may not fully reflect that of physiological membrane rafts (12). It remains nonetheless that DRM preparations represent an excellent system to isolate and identify groups of proteins - eventually associated in complexes - that tend to naturally interact with specific sets of lipids,
The Proteome of Detergent-Resistant Microdomains from Poplar

thereby forming specialized functional units. Their biochemical characterization is therefore most useful to better understand the mode of interaction of specific proteins with sterols and sphingolipids and to gain insight into the protein composition and biological activity of subdomains from the PM.

Plant DRM have been understudied compared to their animal counterpart. Indeed, proteomic studies have been undertaken on DRM preparations from a limited number of plant species only. These include tobacco (13-15), Arabidopsis (16), barrel clover (Medicago truncatula) (17), rice (18), oat and rye (19). These studies essentially based on qualitative or semi-quantitative proteomics led to the identification of hundreds of proteins involved in a large range of mechanisms, functions and biochemical activities (15-19). Depending on the report considered, a variable proportion of the identified proteins could be intuitively linked to DRM and potentially to PM microdomains. However, many proteins that are clearly not related to the PM and its microdomains co-purified with DRM. These comprise for instance soluble proteins from cytoplasmic metabolic pathways, histones, ribosomal, chloroplastic and mitochondrial proteins (15-19). Thus, there is a need to obtain a more confined list of proteins that are specifically enriched in DRM and that define specialized functional structures. This problem can be tackled by undertaking quantitative proteomics, eventually in combination with complementary biochemical approaches. While quantitative techniques have been increasingly applied to the proteomic analysis of complex mixtures of soluble proteins, their exploitation for the characterization of membrane samples remains challenging. As a result, very few studies of plant DRM have been based on truly quantitative methods. For instance, stable isotope labeling combined with the selective disruption of sterol-rich membrane domains by methylcyclodextrin was performed in Arabidopsis cell cultures (20). A similar approach was used to study compositional changes of tobacco DRM upon cell treatment with the signaling elicitor cryptogenin (21). In another study, 64 Arabidopsis proteins were shown
to be significantly enriched in DRM in response to a pathogen-associated molecular pattern protein (22). Altogether, these few quantitative proteomics analyses suggested a role of plant membrane microdomains in signal transduction, as in mammalian cells.

While several reports describe the partial characterization of DRM from higher plants (13-23), there are no data available to date on the protein composition of DRM from a tree species. We have therefore undertaken a quantitative proteomic approach for the characterization of DRM from cell suspension cultures of *Populus trichocarpa*. In addition, earlier work in our laboratory based on biochemical activity assays revealed the presence of cell wall polysaccharide synthases in DRM from poplar (23), which suggests the existence of DRM populations specialized in cell wall biosynthesis. This concept was further supported by similar investigations performed on DRM isolated from the oomycete *Saprolegnia monoica* (24). The comprehensive quantitative proteomic analysis performed here revealed the enrichment in the poplar DRM of specific carbohydrate synthases involved in callose polymerization. Consistent with the role of callose in plant defense mechanisms, additional proteins related to stress responses and signal transduction were found to be specifically enriched in the poplar DRM, together with proteins involved in molecular transport. To date, our report is the only analysis available of the DRM proteome of a tree species based on quantitative proteomics. The specific biochemical properties of the 80 proteins significantly enriched in DRM are described and examined in relation with their localization in membrane microdomains. The relationship between the poplar DRM and molecular transport, signal transduction, stress responses and callose biosynthesis is discussed, supported by a hypothetical model that integrates the corresponding enriched proteins.
EXPERIMENTAL PROCEDURES

Plant Cell Cultures – Poplar (Populus trichocarpa) cell suspension cultures, established as previously described (25), were a gift from Drs Ohlsson and Berglund (KTH Biotechnology). Cells were grown at 24 °C in a modified Murashige and Skoog medium containing sucrose (3%), 2,4-dichlorophenoxyacetic acid (1 mg/l) and kinetin (0.02 mg/l), in a 12 h light/12 h dark regime (25). The cells were harvested in logarithmic growth phase 7-9 days after inoculation and washed twice by vacuum filtration with ice-cold MOPS buffer (0.05 M, pH 7.0).

Isolation of Plasma Membrane and DRM – Isolation of the plasma membrane fraction (PMF) and DRM was performed as described earlier (23). All steps were performed at 4 °C. Typically, 100 g of poplar cells recovered by vacuum filtration were disrupted in 100 mL of homogenization buffer (0.05M MOPS buffer pH 7.0, containing 2 mM EGTA, 2 mM EDTA, 0.33 M sucrose, 1 mM DTT and protease inhibitors from Roche) using a Waring blender. The homogenate was centrifuged at 10,000 x $g$ for 10 min to remove cell debris. The supernatant was filtered through two layers of Miracloth (Calbiochem, Germany) and centrifuged at 50,000 x $g$ for 1 h. The resultant microsomal pellet was resuspended in 6 mL of 5 mM KH$_2$PO$_4$ (pH 7.6) and applied to a two-phase partition system, the final composition of which was 5.8% (w/w) poly(ethylene glycol) 3350 (Sigma), 5.8% (w/w) dextran T-500 (Pharmacosmos A/S, Denmark), 4 mM KCl and 5 mM potassium phosphate (pH 7.6). After mixing 10 times, the mixtures were centrifuged at 500 x $g$ for 10 min and the resulting upper phase was collected and loaded on a newly prepared lower phase. The two-phase separation was repeated and the final upper phase containing the purified PM was diluted 5-fold with MOPS buffer (0.05 M, pH 7.0) and centrifuged at 100,000 x $g$ for 1 h. The PMF pellet was resuspended in 900 µl of MOPS buffer (0.05 M, pH 7.0). The protein concentration was
measured using the Bradford dye-binding assay (Bio-Rad) using bovine serum albumin as a standard.

The DRM were prepared by adding Triton X-100 to the PMF to a final concentration of 1% [detergent-to-protein ratio = 15:1 (w/w)]. After incubation for 30 min on ice, a sucrose solution was added to reach a final concentration of 46%. The preparation was overlaid with a continuous sucrose gradient (45-15 %) and centrifuged at 131,000 x g for 20 h at 4 °C using a swinging rotor (SW27; Beckman). The DRM were recovered as a low-buoyancy white band while the Triton solubilized proteins were collected at the bottom of the tube, i.e. in the 46% sucrose layer (Triton extract, TE). The DRM were diluted with MOPS buffer (0.05 M, pH 7.0) and pelleted by centrifugation at 100,000 x g for 2 h at 4 °C. The DRM and TE proteins were resuspended in MOPS buffer (0.05 M, pH 7.0) and protein content was measured in each sample as described above. A total of four biological replicates were prepared. Out of these, the PMF, DRM and TE from one biological replicate were used for SDS-PAGE (qualitative analysis) while the PMF and DRM from the remaining three replicates were used for the quantitative iTRAQ experiments.

SDS-PAGE Separation and In-gel Hydrolysis of Proteins – SDS-PAGE analysis was repeated on membrane preparations from five independent experiments. All gels exhibited an identical protein profile. Samples were prepared by mixing the PMF, DRM and TE proteins (20 µg) with SDS buffer (3% [w/v] SDS, 75 mM Tris-HCl [pH 6.8], 100 mM DTT, 15% [w/v] glycerol and bromophenol blue). The mixtures were subsequently incubated at 37 °C for 20 min and proteins were separated on 12% SDS polyacrylamide gels. Proteins were stained using Coomassie Blue (Fermentas) and each lane from a single gel was cut from the top to the bottom into 48 bands of similar volume for in-gel digestion (26). Briefly, the gel bands were incubated at 37 °C for 1 h in a solution that consisted of 50% 0.1 M ammonium carbonate, 48.75% acetonitrile, 1% iodoethanol, and 0.25% triethylphosphine (pH 10.0). The liquid was
discarded and the gel pieces were successively dehydrated with 100 µl acetonitrile (ca 5 min),
dried under vacuum, rehydrated in a 30 mM ammonium bicarbonate solution containing 10
ng/µl of sequencing-grade modified trypsin (Promega) and incubated for 16 h at 37°C. The
resulting peptides were extracted, dried, and re-dissolved in 0.1% formic acid for mass
spectrometric analysis (26).

Protein Hydrolysis in Solution and iTRAQ Labeling – The PMF and DRM proteins (100
µg) were solubilized in 0.05 M triethylammonium bicarbonate (TEAB) containing 1% sodium
deoxycholate (SDC; Sigma). Their disulfide bonds were reduced for 1 h at 60 °C in the
presence of 5 mM tris(2-carboxyethyl)phosphine (TCEP) and the resulting free thiol groups
were alkylated at room temperature for 15 min by methyl methanethiosulfonate (MMTS; 10
mM). The proteins were hydrolyzed for 16 at 37 °C in the presence of 5% trypsin in 50 mM
TEAB. The solutions were acidified by adding trifluoroacetic acid (TFA) to a final
concentration of 0.5 % and centrifuged to remove the SDC. The resulting supernatants were
transferred to new tubes and dried under vacuum. The dried peptides from the PMF and DRM
samples were dissolved in 100 µL of a mixture consisting of 25% of 250 mM TEAB and 75%
(v/v) ethanol, and transferred to different vials containing the different iTRAQ reagents (114-
117; AB SCIEX). After 1 h incubation at room temperature, the reaction was stopped by
adding 100 µL of Milli-Q water. The iTRAQ-labeled PMF and DRM samples were pooled
and the mixtures were dried under vacuum. The iTRAQ labeling of the peptides from the
other two biological replicates was performed in the same conditions except that the labels
were inverted to reduce bias between samples.

Strong Cation-Exchange (SCX) Fractionation of the iTRAQ-Labeled Peptides – The dried
iTRAQ-labeled peptides were resuspended in 3 ml of sample loading buffer (10 mM
ammonium formate, 20% acetonitrile, pH 3.0) and loaded on a 1-mL Nuvia™S cartridge
(Bio-Rad; prepared according to the manufacturer’s instructions) at 0.5 mL/min using a
syringe pump. After sample loading, the cartridges were washed by 5 mL of sample loading buffer at 0.5 ml/min, followed by elution at the same flow rate with consecutive 1.5-mL ammonium formate salt plugs at pH 3.0 (30, 50, 80, 100, 125, 150, 175, 200, 225, 250, 275, 300, 325, 350 and 400 mM, respectively, in 20% acetonitrile). The eluent from each salt plug was dried using a SpeedVac centrifugal vacuum concentrator and the peptides were purified on a PepClean C-18 column (Thermo Scientific) prior to mass spectrometry (MS) analysis.

Nano-LC–MS/MS Analysis of the SCX Fractions – Peptide analysis was performed by reverse-phase LC-ESI-MS/MS, using a nanoACQUITY Ultra Performance Liquid Chromatography system coupled to a Q-TOF mass spectrometer (Xevo Q-TOF, Waters Corporation). The purified SCX fractions were resuspended in 0.1% TFA, loaded on a C18 trap column (Symmetry 180 μm × 20 mm, 5 μm; Waters, Milford, MA) which was then washed with 1% (v/v) acetonitrile, 0.1% (v/v) formic acid at 15 μl/min for 10 min. The samples eluted from the trap column were separated on a C18 analytical column (75 μm × 100 mm, 1.7 μm; Waters, Milford, MA) at 350 nL/min, using 0.1% formic acid as solvent A and 0.1% formic acid in acetonitrile as solvent B, in a stepwise gradient: 0.1-10% B (0-10 min), 10-30% B (10-110 min), 30-40% B (110-120 min), 40-85% B (120-125 min), 85% B (125-130min), 85-0.1% B (130-135 min). The eluting peptides were sprayed in the mass spectrometer (capillary and cone voltages set to 4 kV and 35 V, respectively) and MS/MS spectra were acquired using an automated data-directed switching between the MS and MS/MS modes using the instrument software (MassLynx V4.0 SP4). The five most abundant signals of a survey scan (350-1500 m/z range, 0.9 s scan time) were selected by charge state, and collision energy was applied accordingly for sequential MS/MS fragmentation scanning (50-1800 m/z range, 0.9 s scan time).

Data Processing, Protein Identification and Quantification – An extensive search scheme was used to rigorously profile the MS data following the strategy presented in Fig. 1. The MS
raw data files were processed using Mascot Distiller (version 2.4.3.2, MatrixScience). The resulting mgf files were converted into the mzXML file format using msconvert (27) and back to the mgf format using mzXML2Other (28) to assign proper titles to each spectrum. The mzXML files were searched by MyriMatch (29) (version 2.1.120) and X!Tandem (30) (version 2011.12.01.1 - LabKey, Insilicos, ISB) using the Populus protein database (Populus version 3.0; 73,013 entries; http://www.phytozome.net/) and the following settings: trypsin specific digestion with two missed cleavages allowed, peptide tolerance of 80 ppm, fragment tolerance of 0.2 Da, iTRAQ 4-plex for peptide N-t and Lys as fixed modifications and, in variable mode, iTRAQ 4-plex on Tyr, oxidized Met and methylthio on Cys. For the SDS-gel samples, all search parameters were as above, except that ethanoylated Cys and oxidized Met were used as fixed and variable modifications, respectively. The final mgf outputs were submitted to a local Mascot (Matrix Science, version 2.3.1) server using the same search settings as indicated above. The Mascot search results were exported as Mascot dat files and converted to pep.xml using Mascot2XML (31). The results from the Mascot, MyriMatch and X!Tandem search engines were validated by PeptideProphet (32) using the parametric (Mascot) and semi-parametric (MyriMatch and X!Tandem) models. For quantitative analysis, all iTRAQ reporter ion intensities were extracted using the TPP tool Libra (http://tools.proteomecenter.org) and the isotopic correction factors from the iTRAQ reagent manufacturer. Normalization of iTRAQ channels was performed by summing all intensities of reporter ions in each iTRAQ channel (for peptides above the Libra probability cutoff) and equalizing each channel contribution by dividing individual reporter ion intensities by the corresponding channel-specific correction factor. All pep.xml files obtained from PeptideProphet were combined using iProphe t (33). A protein list was assembled using ProteinProphet (34) and the final protein ratios were calculated using Libra. For the samples from SDS-PAGE, data processing was identical but without the iTRAQ related steps. In all
searches a concatenated target-decoy database-search strategy was used to check the false positive rate, which was found to be less than 1% in all cases.

Peptide sequences were exported for each protein, with a protein and peptide probability cutoff of 0.95. Peptides matching two or more proteins (shared peptides) were excluded from the analysis. Proteins with no unique peptides, i.e. only identified by shared peptides, were also excluded. A protein was considered as identified if it contained at least one unique peptide. Only proteins identified by two or more unique peptides were used for quantification. The method of Ross et al. (35) was used for statistical analysis of the quantitative data. Briefly, the DRM/PMF ratio of each protein was calculated for each of the three biological replicates (supplemental Table S2) and log2 transformed to obtain a normal distribution. The values were then normalized to the median log values and global means and standard deviations were calculated for each biological replicate. Proteins whose average ratios fell outside a standard deviation of ±1 from the global mean in at least two out of three biological replicates were considered significantly enriched.

**Prediction of Topology and Post-translational Modifications** – The sequences of all identified proteins were used for the prediction of transmembrane domains (TMD), sites of glycosylphosphatidylinositol (GPI) modification, myristoylation, and palmitoylation. The number of TMD was determined by the HMMTOP program (http://www.enzim.hu/hmmtop/). GPI modification, N-terminal myristoylation and palmitoylation sites were analyzed using the PredGPI predictor (http://gpcr.biocomp.unibo.it/predgpi/pred.htm), Plant-Specific Myristoylation Predictor (http://plantsp.genomics.purdue.edu/myrist.html) and CSS-Palm 3.0 (http://csspalm.biocuckoo.org/online.php; with high threshold) algorithms, respectively. Theoretical molecular mass and isoelectric points (pI) were determined by the Protein Molecule Weight and the Isoelectric Point Calculator (http://www.endmemo.com/bio/). Gene ontology information was retrieved from TAIR (www.arabidopsis.org).
RESULTS

Following our previous work on the isolation of DRM from poplar cell suspension cultures and enzymatic assays (23), we have undertaken a proteomic approach for profiling the composition of DRM and gaining further insight into the function of these microdomains. In the first instance, a gel-based qualitative analysis was performed on the PMF, DRM and TE fractions to obtain an extended list of proteins present in each of these fractions. This was followed by an in-depth quantitative analysis on three biological replicates to determine the subset of proteins that are truly enriched in DRM.

*Qualitative Profiling of DRM* – A total of 2396 different proteins were identified from PMF, DRM and TE altogether (supplemental Table S1). The analysis of the PMF, DRM and TE in-gel hydrolysates allowed the identification of a total of 917, 267 and 342 proteins in each of these 3 samples, respectively (Fig. 2 and supplemental Table S1). Out of these, 30 only were common to the DRM and TE (Fig. 2), which demonstrates the efficiency of the Triton X-100 treatment for separating proteins associated to DRM from the rest of the plasma membrane-bound proteins. As expected, a significantly higher proportion of proteins were common to PMF and DRM on one hand [166 (=141 + 25)] and to PMF and TE [177 (=152 + 25)] on the other hand (Fig. 2). In addition, each of the three samples contained proteins that were not identified in the two other samples, *i.e.* 599, 96 and 160 proteins were only detected in the PMF, DRM or TE, respectively (Fig. 2).

Proteins from the DRM, PMF and TE were classified based on their predicted cellular localization (Fig. 3A, B and supplemental Fig. S1A) and the biological processes in which they are involved (Fig. 3C, D and supplemental Fig. S1B). Interestingly, 90% of the 267 DRM proteins identified after in-gel hydrolysis were classified as membrane-bound proteins, while only minor proportions of these (~5%) were predicted to be non-membrane proteins.
located in the cytoplasm or more specifically associated to ribosomes (Fig. 3A). The rest of the identified DRM proteins were either extracellular (2%) or had an unknown cellular localization (3%). Both the PMF and TE contained a significantly lower proportion of identified proteins predicted to be associated with cell membranes. Indeed, the percentage of proteins that are not membrane bound was of ~40% and 60% in the PMF and TE, respectively (Fig. 3B and supplemental Fig. S1A). These data show that the PM were not purified to homogeneity in the PMF and that the highest proportion of non-membrane proteins that initially co-segregate with the plasma membranes are recovered in TE. Thus, in addition to allowing the enrichment of DRM, the Triton X-100 treatment is an efficient step for removing contaminating soluble proteins from membranous fractions.

The proteins identified in DRM were associated to transport (43%), metabolism (19%), response to stress (13%) and signal transduction (11%) (Fig. 3C). Each fraction also contained a relatively small proportion (7-12%) of unclassified proteins whose function is unknown (Fig. 3C, D and supplemental Fig. S1B). The proportions of the different classes of proteins were moderately modified in PMF, the most important differences being an increase in proteins involved in response to stress (20%) and a decrease in transporters (30%) compared to the DRM (Fig. 3D). The most distinguishable fraction was TE, which, as opposed to DRM and PMF, contained a high proportion (34%) of proteins involved in protein biosynthesis. These are related to ribosomes, which were already identified as more abundant in TE (supplemental Fig. S1B). In addition, TE contained a significantly lower proportion of proteins involved in transport (19%) and signal transduction (2%) (supplemental Fig. S1B).

Altogether, these data most likely reflect an initial co-purification of endoplasmic reticulum with PM, whose components, including ribosomes, segregate preferentially in TE after the detergent treatment of PMF. However, we cannot completely rule out the possibility that some of the proteins we have identified may occur in multiple cellular compartments.
SDS-PAGE Profiles and Quantitative Proteomic Analysis – SDS-PAGE analysis revealed that the protein profiles of the DRM, PMF and TE fractions are clearly distinguishable (Fig. 4). Many PMF proteins were preferentially extracted by the detergent (Triton X-100) and thus expected to be essentially recovered in the high-density TE fraction. Consistent with this, DRM exhibited a much simpler profile than PMF (Fig. 4). In addition, SDS-PAGE analysis revealed that at least 14 protein bands seem to be significantly enriched in the DRM, as judged by their intensity after Coomassie blue staining (arrowheads in Fig. 4). The iTRAQ-based quantitative analysis revealed that the actual total number of proteins enriched in DRM compared to PMF was 80 (Table I). Gene ontology analysis revealed that 77 of them are predicted to be membrane-bound, while only 3 have an unidentifiable cell location (Table I). Functional classification of the proteins enriched in DRM shows that they can be grouped in 3 different main families: 50% are transporters, 19% are related to stress responses and 20% are involved in signal transduction (Fig. 5). The remaining 11% cannot be confidently linked to any function. The latter group corresponds to either uncharacterized proteins or to proteins that do have an annotation, but whose molecular function is unknown. Although data from cell suspension cultures may not fully reflect the situation in intact plant tissues, the remarkably simple distribution of the enriched proteins into 3 main functional families most likely reflects a specialization of the isolated DRM in cell surface related processes, especially transport, responses to stress and signal transduction. Furthermore, our data are consistent with other reports describing the qualitative protein composition of DRM isolated from plant tissues (13, 14, 17, 19).

Biochemical Properties of Proteins Enriched in DRM – As expected, a large majority (95%) of the 80 DRM-enriched proteins are predicted to contain up to 16 TMD and/or membrane-anchoring acylation sites. Only a minority (4 out of 80) contain neither membrane spanning domains nor post-translational modifications responsible for attachment to
membranes. Of all enriched proteins, about 64% are predicted to contain at least 2 TMD while 15% exhibit one such domain only (Table I). Most interestingly, proteins enriched in DRM are characterized on average by a higher number of TMD compared to all PM proteins (Fig. 6A). In addition, their TMD have a significantly higher length than those of PM proteins (Fig. 6B). The molecular weights and isoelectric points of total PM and DRM enriched proteins were also analyzed. Proteins below 18 kDa were not present in DRM (Fig. 6C). Instead, DRM contained a higher proportion of high molecular weight proteins, compared to PMF (Fig. 6C). Approximately 59% of the DRM-enriched proteins exhibited predicted pIs in the range of 8-10 while the remaining 41% were less alkaline (pI 4-7) (Fig. 6D). Comparison with PMF proteins revealed that acidic proteins (pI <6) are more abundant in PMF whereas basic proteins (pI >8) are present in higher proportions in DRM. These features are comparable to those reported for DRM proteins from tobacco (15).

Bioinformatics tools were used to predict the number of TMD and the type of membrane-anchoring post-translational modifications that the DRM-enriched proteins may contain (see Experimental Procedures). The data suggest that most of the 21% DRM-enriched proteins that are devoid of membrane spanning domains contain acylation sites (Table I). The in silico analyses also indicate that about 54% of DRM-enriched proteins predicted to be associated to the membrane contained at least one TMD and an acylation site. Among the 56 proteins predicted to be acylated, 5 contained both myristoylation and palmitoylation sites while all others contained palmitoylation as the single predicted modification. Altogether these bioinformatics predictions tend to confirm that the isolated DRM exhibit the expected enrichment in transmembrane and palmitoylated proteins.
DISCUSSION

Suspension cultures of tree cells represent a convenient alternative system to resilient woody tissues for the isolation of membrane structures and their proteomic characterization. *Populus* is a well-established model system for woody plants and, as such, it provides an opportunity to study processes for which herbaceous models are less adapted (36). In order to shed light on the identity and function of proteins specifically enriched in plasma membrane DRM from a tree species, we have undertaken the proteomic characterization of such microdomains from *Populus trichocarpa* cell suspension cultures by using both qualitative and quantitative approaches (Fig. 1). First, we qualitatively analyzed the composition of PMF and DRM after protein separation by SDS-PAGE (Fig. 4). This allowed the identification of hundreds of proteins in each fraction by mass spectrometry (Fig. 2). Sodium deoxycholate was used for the solubilization of the PMF and DRM proteins prior to their partial enzymatic hydrolysis in solution. This detergent has the advantage of increasing the solubility of membrane proteins, which is accompanied by an enhanced efficiency of trypsin and a higher recovery of hydrophobic peptides, while being easy to remove by acidification after the proteolysis step (37). Peptides/proteins were quantified using the iTRAQ approach, which allows the concomitant analysis of proteins in up to 8 samples, limits sample complexity and reduces experimental variability between samples (35, 38, 39).

Our strategy has allowed the identification of a total of 2396 unique proteins in the poplar samples altogether, out of which nearly 48% (1151 proteins) are predicted to contain at least one TMD (supplemental Table 1). These numbers are significantly higher compared to the only proteomics study available on poplar PM (8). Indeed, in the latter case only 22% of the total 956 identified proteins were predicted to contain at least one TMD. Similar analyses on *Arabidopsis* PM revealed that ~38% (4) and 51% (40) of the total identified proteins contain one or more putative TMD. Interestingly, nearly 80% of the 80 proteins enriched in poplar
The Proteome of Detergent-Resistant Microdomains from Poplar

DRM have one or more predicted TMD (Table I). In tobacco cells, 59% of the DRM identified proteins showed one or more putative TMD (15), similar to the percentage (~60%) reported in DRM from oat and rye (19). Further, in our study, ~46% of all the DRM-enriched proteins exhibit more than 7 TMD. This is significantly higher than in the case of *Arabidopsis* (~29%) (40) and tobacco (40%) (15). As reported earlier in tobacco (15), we observe a higher average number of amino acids per TMD in the DRM-enriched proteins compared to the total proteins from the PM (Fig. 6B). Altogether, our data further support the earlier suggestion that DRM are thicker than the detergent-soluble parts of the PM and, consequently, that they tend to contain a higher proportion of proteins with longer TMD (41). In addition to TMD, acylation and glypilation are involved in anchoring proteins in membrane structures. Palmitoylation is a reversible type of post-translational modification responsible for membrane association of proteins involved in multiple cell surface processes (42). The observed enrichment of the poplar DRM in palmitoylated proteins is consistent with the functional specialization of such microdomains in signal perception and transduction as well as in transport at the PM. GPI-anchored proteins represent another type of proteins expected to be enriched in DRM (43). It is noteworthy that our analyses of the 80 proteins enriched in DRM revealed that 2 proteins only are predicted to contain a GPI post-translational modification (Table I). This might be due to the natural low abundance of such proteins in biological membranes and the consequent need to use an enrichment protocol specific for GPI proteins to facilitate their detection. Indeed, mass spectrometric identification of GPI-anchored proteins from plant cells is greatly facilitated by using a specific Triton X-114 based two-phase partitioning step, combined with the action of phosphatidylinositol phospholipase C, to specifically enrich and release the protein moieties from their GPI-anchors (44, 45). Even when such specific approaches are used, the total number of GPI-anchored proteins identified typically corresponds to a fraction only of the actual GPI-modified proteins, as
illustrated by the relatively low number (<50) of GPI-anchored proteins experimentally identified from total plasma membranes of Arabidopsis cell suspension cultures (44).

Quantitative proteomic analyses revealed that the poplar DRM-enriched proteins correspond to 3 main functional categories, namely transport (50%), responses to stress (19%) and signal transduction (20%) (Fig. 5). The relevance of their presence in DRM is discussed below.

Remorins – Remorins have been previously identified in DRM from tobacco (15), barrel clover (17), oat and rye (19) as well as in the proteome of Arabidopsis plasmodesmata (46). In tobacco, remorins accumulate preferentially within the cytosolic leaflet of thick (~70 nm) plasmodesmatal membrane domains associated with plant defense mechanisms (47-49). Furthermore, quantitative proteomics in Arabidopsis involving metabolic labeling and specific disruption of sterol-rich membrane domains by methyl-β-cyclodextrin strongly suggested that remorins are sterol-dependent proteins (20). Altogether, these data and our finding that remorins are also enriched in the poplar DRM (Table I) further support the concept that remorins are functional markers of this type of microdomains.

Intracellular Trafficking – Six proteins involved in membrane trafficking were enriched in the poplar DRM (Table I). Out of these, one is a secretory carrier membrane protein (SCAMP) containing 4 putative TMD (Table I). In animal cells, SCAMPs are post-Golgi integral membrane proteins mediating endocytosis (50). Interestingly, a rice homolog of an animal SCAMP was found to be targeted to the PM, trans-Golgi network and early endosomes when expressed in tobacco (51). Although plant SCAMPs have never been linked to DRM in any proteomics studies, it is tempting to speculate that SCAMP-mediated endocytic pathways involve specific membrane microdomains. It remains however to determine whether SCAMP are located in such thick domains of the PM by imaging and other complementary techniques. A Q-SNARE (syntaxin 71), a phospholipid translocase (ALA3)
and ALA-interacting protein (ALIS1) were also enriched in the poplar DRM (Table I). This type of proteins participates in intracellular membrane fusion, vesicle formation and trafficking (52, 53). The association of ALIS1 to ALA3 is required for phospholipid translocase activity and the resulting complex is a key component of the Golgi secretory machinery (53). It is noteworthy that DRM proteomic studies in tobacco (15), rice (18), oat and rye (19) have also reported the presence of similar proteins involved in trafficking.

Transport Proteins – Several types of transporters, including ABC transporters, aquaporins, sugar, metal, inorganic and organic solute transporters and ATPases were significantly enriched in the poplar DRM (Table I). The occurrence of the same classes of transport proteins has also been reported in DRM from other plants such as tobacco (15) and Arabidopsis (16), and in monocots such as rice (18), oat and rye (19). Out of the 8 ATPases enriched in the poplar DRM, one was a Ca\(^{2+}\)-ATPase while others belonged to the P\(_{3}\)-type of ATPases (Table I). P-ATPases transport different compounds across the PM upon ATP hydrolysis, including ions and phospholipids (54). As opposed to other studies in tobacco (15), Arabidopsis (16) and the monocots oat and rye (19), no V-type ATPase was identified in the poplar DRM. Interestingly, the relative abundance of P-type H\(^{+}\)-ATPases was increased in Arabidopsis DRM during cold acclimation, accompanied with a decrease in V-type H\(^{+}\)-ATPase subunits (55), consistent with the observation that the expression of H\(^{+}\)-ATPase genes changes as a result of abiotic and biotic stresses (56). Similarly, the expression of plant Ca\(^{2+}\)-ATPases has been associated with salt stress (57) and in maintaining calcium homeostasis within the cell (58).

Aquaporins are members of the PM intrinsic protein family and facilitate the movement of water through membranes. The level of some of them is increased during cold acclimation in Arabidopsis (55). Interestingly, 4 such proteins were enriched in the poplar DRM (Table I). Plant ABC transporters have been associated for instance to auxin transport, lipid catabolism,
disease resistance and the function of the stomata (59). Four proteins from ABC transporter subfamilies, i.e. multidrug resistance (MDR, 3 proteins) and pleiotropic drug resistance (PDR, 1 protein), were enriched in the poplar DRM (Table I). The *Arabidopsis* AtPDR protein is localized in the PM and involved in the removal of toxic compounds (Pb) from the cytoplasm (60) while AtMDR4 is an auxin transporter (61). Several of the poplar DRM-enriched transporters were also enriched in DRM from *Arabidopsis* in response to flagellin, a pathogen-associated molecular pattern (PAMP) protein (22). In addition, the expression of some sugar transporters is affected as a response to biotic and abiotic stresses (62). Altogether, these observations and our data show that, even for proteins that are typically classified in functional groups other than “response to stress”, a link to stress response can be established.

*Signal Transduction and Response to Stress* – Rop/Rac GTPases are important regulators of signal transduction in plants (63). It has been suggested that acylation regulates ROP signaling in PM microdomains (64). Two of this type of proteins, ROP 6 and 10, were enriched in the poplar DRM (Table I). In *Arabidopsis*, ROP10 is a PM-localized protein that negatively regulates the sensitivity of stress-responsive genes to abscissic acid (65). Unlike its inactive prenylated form, which was recovered in detergent-soluble fractions, the active form of ROP6 was specifically acylated and localized in the *Arabidopsis* DRM (64). Interestingly, it was also shown that the rice GTPase Rac1 and its effector RACK1A segregate in DRM upon elicitation with chitin and are involved in the plant innate immunity (18). In addition to Rop/Rac GTPases, the poplar DRM were also enriched in other types of proteins that are directly involved in the signaling cascade, such as leucine-rich repeat protein kinases and receptor kinases (Table I).

In plants, calcium is an important secondary messenger involved in the regulation of stress-signaling pathways (66). Consistent with the function of DRM in these processes, many
proteins dependent on calcium and calmodulin for their activity were significantly enriched in the DRM (Table I). Examples are a calcium-dependent protein kinase, which plays an important role in plant defense responses in tobacco (67) and calcium-dependent lipid binding proteins, which act as repressors of abiotic stress response in Arabidopsis (68).

Phospholipase D (PLD) and diacylglycerol kinase (DGK), both involved in the biosynthesis of phosphatidic acid (PA) (69), were enriched in the poplar DRM (Table I). PA acts as a second messenger and is involved in many biotic and abiotic stress responses (70). Protein phosphatase 2C (PP2C), which is one of the main targets of PA (70), was also enriched in the poplar DRM (Table I). Likewise, PLD clusters at the site of infection of the PM of rice cells attacked by the pathogenic bacterium Xanthomonas oryzae (71). In addition to being involved in responses to pathogens, phospholipases are associated to abiotic stress, as exemplified by the increased freezing tolerance of Arabidopsis upon overexpression of the PM-associated PLDδ (72).

Callose Synthases – The poplar DRM contained several isoforms of glucan synthase-like (GSL) proteins, namely GSL 5, 8, 10 and 12 (Table I), which are involved in different biological processes associated to callose deposition at the surface of the PM. These polytopic proteins were among the most enriched in the DRM, which suggests a specialization of the microdomains in callose biosynthesis. Callose deposition and GSL are typically involved in specialized physiological processes, as demonstrated for GSL 5 (pollen development and fertility) (73), GSL 8 and 10 (male gametophyte development, cytokinesis and plant growth) (74, 75), and GSL 12 (plasmodesmata aperture and associated regulation of molecular trafficking) (76). In addition, GSL 5 has been reported to be involved in wound and papillary callose deposition (77), thereby having a dual function in plant development (73) and stress response (77). Aniline blue staining of callose in the poplar cells used for our proteomics analyses revealed a punctate type of pattern for callose deposition (supplemental Fig. S2,
A similar specific pattern has been observed in *Arabidopsis* cell suspension cultures stressed by plasmolysis or cellulase treatments (78). Our data suggest that the observed callose deposition in the poplar cell suspension cultures (supplemental Fig. S2) is a result of stress, although no specific treatment was used in our experiments to trigger a stress response. The observed punctate pattern reflects localized deposition sites at the surface of the plasma membrane rather than a homogeneous deposition of callose throughout the cell walls. This is consistent with the association and enrichment of callose synthases in defined lateral patches in the plasma membrane, *i.e.* membrane microdomains (DRM). Further to these observations, callose synthase assays performed on the isolated DRM confirmed the occurrence of active enzymes in the microdomains. Indeed, the poplar DRM prepared here exhibited a specific callose synthase activity similar to that reported in our earlier work (data not shown) (23). Thus, it can be concluded that some, if not all, of the callose synthases identified here are functional in the isolated microdomains. Altogether our data indicate that callose synthase activity is confined and enriched in poplar DRM and that callose deposition in the cell cultures most likely arises from stress. It can be inferred that the isolated DRM reflect a natural interaction of the identified callose synthases (and the other proteins identified) with the specific types of structural lipids that are typically expected to be enriched in DRM, namely sterols, sphingolipids and phospholipids that preferentially contain saturated fatty acids (9, 10, 23). Interestingly, plasmodesmata have been shown to occur in cell suspension cultures (78, 79). There is also substantiated evidence that callose synthases are plasmodesmal proteins associated to several physiological and developmental processes, cell-to-cell communication and biotic and abiotic stress responses (80, 81, and references therein). Thus, consistent with the involvement of callose synthase activity in these processes, we hypothesize that some of the DRM isolated here may arise from plasmodesmatal structures. The relationship between callose synthase and the other DRM-enriched proteins identified
The Proteome of Detergent-Resistant Microdomains from Poplar

Here is presented in an integrated hypothetical model (Fig. 7). Although additional work is needed to confirm the actual function of some of the identified proteins, the model reflects the general proposed function of the isolated microdomains in stress response and connects the different categories of proteins identified in the poplar DRM.
REFERENCES


The Proteome of Detergent-Resistant Microdomains from Poplar

tion in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. Mol. Cell. Proteomics 3, 1154-1169


The Proteome of Detergent-Resistant Microdomains from Poplar


PGP4, an ATP-binding cassette P-glycoprotein, catalyzes auxin transport in *Arabidopsis thaliana* roots. *Plant Cell* 17, 2922-2939


The Proteome of Detergent-Resistant Microdomains from Poplar


ACKNOWLEDGMENTS

The authors thank Drs Ohlsson and Berglund (KTH Biotechnology) for the generous gift of the poplar cell suspension cultures, which were established in 2007, as described earlier (25). Confocal microscopy was performed at the “Imaging Facility of Stockholm University” (IFSU).

FOOTNOTES

Financial support was from the Swedish Centre for Biomimetic Fibre Engineering (grant from the Swedish Foundation for Strategic Research to V.B.) and the KTH Advanced Carbohydrate Materials Consortium (grant from the Swedish Research Council FORMAS to V.B.).
FIGURE LEGENDS

FIG. 1. Experimental set-up and strategy used for data analysis. PMF, plasma membrane fraction; DRM, detergent-resistant microdomains; TE, Triton extract; iTRAQ, isobaric tags for relative and absolute quantitation; SCX, strong cation-exchange chromatography.

FIG. 2. Qualitative proteomic analysis of PMF (plasma membrane fraction), DRM (detergent-resistant microdomains) and TE (Triton extract). The Venn diagram represents the number of proteins identified from SDS-PAGE of PMF, DRM and TE. In total, 917, 267 and 342 proteins were identified by MS analysis of PMF, DRM and TE, respectively.

FIG. 3. Predicted cellular localization (A, B) and functional classification (C, D) of proteins identified after SDS-PAGE analysis of DRM and PMF. (A and C) DRM (detergent-resistant microdomains); (B and D) PMF (plasma membrane fraction). Gene ontology information was retrieved from TAIR (www.arabidopsis.org).

FIG. 4. SDS-PAGE analysis of proteins present in DRM (detergent-resistant microdomains), TE (Triton extract) and PMF (plasma membrane fraction). A total of 20 μg protein was loaded in each lane. Each lane of the Coomassie-blue-stained gel was cut into 48 bands as shown on the left side of the picture. Arrowheads point to protein bands of a higher intensity in DRM compared to TE and PMF.
FIG. 5. Functional classification of the 80 proteins enriched in the poplar DRM based on gene ontology prediction. Gene ontology information was retrieved from TAIR (www.arabidopsis.org).

FIG. 6. Classification of DRM-enriched (green bars) and total plasma membrane proteins (blue bars) based on their number of transmembrane domains (TMD) (A), average number of amino acids in TMD (B), molecular mass (C) and isoelectric point (D).

FIG. 7. Integrated hypothetical model representing the different proteins enriched in the poplar DRM and the biological processes in which they are involved. Proteins whose names are highlighted in blue were shown to be enriched in plasma membrane DRM (grey patches) together with other proteins listed in Table I. All proteins enriched in DRM are markers of signal transduction, molecular transport and callose biosynthesis and known to be involved in biotic and/or abiotic stress. Vesicles carrying proteins involved in endocytosis and exocytosis and cell wall biosynthetic enzymes (callose synthases or GSL) are represented by green circles. The represented cascade of events leads to the release of calcium on the cytoplasmic side of the plasma membrane, which is required for the activation of callose synthase as a final response to stress (80). The enrichment of callose synthase in DRM suggests a preferential deposition of callose plugs at the surface of plasma membrane microdomains, possibly in specialized plasmodesmatal structures and/or other sites of the plasma membrane. DAG, diacylglycerol; DGK, diacylglycerol kinase; ER, endoplasmic reticulum; GSL, glucan synthase-like/callose synthase; IP3, inositol-3-phosphate; PIP, phosphatidyl-inositol-phosphate; PL, phospholipid; PLC, phosphatidyl-inositol phospholipase.
The Proteome of Detergent-Resistant Microdomains from Poplar

C; PLD, phospholipase D; PM, plasma membrane; SCAMPS, secretory carrier membrane proteins; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor.
### Table I

List of proteins enriched in DRM compared to PMF. CL, cellular location; Mem, membrane-bound; Unk, unknown; DRM/PMF iTRAQ ratio, quantitative ratio between DRM and PMF based on iTRAQ; Seq. Cov., percent sequence coverage; (pep.), number of unique peptides identified; PTM, predicted post-translational modifications; Pal, palmitoylation; Myr, myristoylation; GPI, glycosylphosphatidylinositol; TMD, number of transmembrane domains

<table>
<thead>
<tr>
<th>Accession No.</th>
<th>Closest ATG</th>
<th>Description</th>
<th>CL</th>
<th>Functional Category</th>
<th>DRM/PMF iTRAQ ratio</th>
<th>Seq. Cov. (pep.)</th>
<th>PTM</th>
<th>TMD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potri.012G047100.1</td>
<td>AT3G17980.1</td>
<td>Calcium-dependent lipid-binding protein</td>
<td>Unk</td>
<td>Response to Stress/Defence</td>
<td>1,38</td>
<td>30.2 (10)</td>
<td>Pal, Pal</td>
<td>1</td>
</tr>
<tr>
<td>Potri.012G108200.1</td>
<td>AT5G07300.1</td>
<td>Calcium-dependent phospholipid-binding protein</td>
<td>Mem</td>
<td>Response to Stress/Defence</td>
<td>2,09</td>
<td>51.4 (10)</td>
<td>Pal</td>
<td>2</td>
</tr>
<tr>
<td>Potri.001G012000.1</td>
<td>AT3G07160.1</td>
<td>Glucan synthase-like 10</td>
<td>Mem</td>
<td>Response to Stress/Defence</td>
<td>1,88</td>
<td>33.8 (35)</td>
<td>Pal</td>
<td>14</td>
</tr>
<tr>
<td>Potri.001G011900.1</td>
<td>AT3G07160.1</td>
<td>Glucan synthase-like 10</td>
<td>Mem</td>
<td>Response to Stress/Defence</td>
<td>2,13</td>
<td>6 (7)</td>
<td>Pal</td>
<td>16</td>
</tr>
<tr>
<td>Potri.001G012200.1</td>
<td>AT5G13000.1</td>
<td>Glucan synthase-like 12</td>
<td>Mem</td>
<td>Response to Stress/Defence</td>
<td>2,00</td>
<td>24.3 (24)</td>
<td>Pal</td>
<td>14</td>
</tr>
<tr>
<td>Potri.013G131300.1</td>
<td>AT4G03550.1</td>
<td>Glucan synthase-like 5</td>
<td>Mem</td>
<td>Response to Stress/Defence</td>
<td>2,06</td>
<td>27.1 (37)</td>
<td>Pal</td>
<td>16</td>
</tr>
<tr>
<td>Potri.001G078100.1</td>
<td>AT5G62740.1</td>
<td>Hypersensitive-induced reaction 4</td>
<td>Mem</td>
<td>Response to Stress/Defence</td>
<td>2,16</td>
<td>71.9 (11)</td>
<td>Pal</td>
<td>0</td>
</tr>
<tr>
<td>Potri.002G157700.1</td>
<td>AT3G61260.1</td>
<td>Remorin</td>
<td>Mem</td>
<td>Response to Stress/Defence</td>
<td>1,75</td>
<td>48.2 (8)</td>
<td>Pal</td>
<td>0</td>
</tr>
<tr>
<td>Potri.014G081300.1</td>
<td>AT3G61260.1</td>
<td>Remorin</td>
<td>Mem</td>
<td>Response to Stress/Defence</td>
<td>1,75</td>
<td>48.2 (8)</td>
<td>Pal</td>
<td>0</td>
</tr>
<tr>
<td>Potri.008G182900.1</td>
<td>AT4G35230.1</td>
<td>BR-signaling kinase 1</td>
<td>Mem</td>
<td>Signal Transduction</td>
<td>1,48</td>
<td>6.3 (2)</td>
<td>Pal</td>
<td>2</td>
</tr>
<tr>
<td>Potri.002G249300.1</td>
<td>AT3G25290.1</td>
<td>Auxin-responsive family protein</td>
<td>Mem</td>
<td>Signal Transduction</td>
<td>5,13</td>
<td>25.2 (6)</td>
<td>Pal</td>
<td>5</td>
</tr>
<tr>
<td>Potri.001G003400.1</td>
<td>AT4G04720.1</td>
<td>Calcium-dependent protein kinase 21</td>
<td>Mem</td>
<td>Signal Transduction</td>
<td>1,75</td>
<td>45.1 (19)</td>
<td>Myr, Pal</td>
<td>1</td>
</tr>
<tr>
<td>Potri.009G052700.1</td>
<td>AT5G12480.1</td>
<td>Calmodulin-domain protein kinase 7</td>
<td>Mem</td>
<td>Signal Transduction</td>
<td>1,56</td>
<td>16.7 (7)</td>
<td>Myr, Pal</td>
<td>1</td>
</tr>
<tr>
<td>Potri.018G096700.1</td>
<td>AT2G18730.1</td>
<td>Diacylglycerol kinase 3</td>
<td>Mem</td>
<td>Signal Transduction</td>
<td>1,48</td>
<td>30.2 (8)</td>
<td>Pal</td>
<td>1</td>
</tr>
<tr>
<td>Potri.006G174700.1</td>
<td>AT4G30340.1</td>
<td>Diacylglycerol kinase 7</td>
<td>Mem</td>
<td>Signal Transduction</td>
<td>1,3</td>
<td>21.3 (6)</td>
<td>Pal</td>
<td>1</td>
</tr>
<tr>
<td>Potri.018G074300.1</td>
<td>AT2G26730.1</td>
<td>Leucine-rich repeat protein kinase</td>
<td>Mem</td>
<td>Signal Transduction</td>
<td>1,61</td>
<td>36.6 (15)</td>
<td>Pal</td>
<td>1</td>
</tr>
<tr>
<td>Gene Accession</td>
<td>Gene Symbol</td>
<td>Gene Name</td>
<td>Function</td>
<td>Membrane Localization</td>
<td>Transporter</td>
<td>Transport Function</td>
<td>Palmitoylation</td>
<td>Comments</td>
</tr>
<tr>
<td>---------------</td>
<td>-------------</td>
<td>-----------</td>
<td>----------</td>
<td>------------------------</td>
<td>-------------</td>
<td>---------------------</td>
<td>--------------</td>
<td>----------</td>
</tr>
<tr>
<td>Potri.016G061500.1</td>
<td>AT1G53440.1</td>
<td>Leucine-rich repeat protein kinase</td>
<td>Mem</td>
<td>Signal Transduction</td>
<td>1.25</td>
<td>30.2 (19)</td>
<td>Pal</td>
<td>2</td>
</tr>
<tr>
<td>Potri.005G043700.1</td>
<td>AT1G05700.1</td>
<td>Leucine-rich repeat receptor kinase</td>
<td>Mem</td>
<td>Signal Transduction</td>
<td>1.8</td>
<td>50.8 (17)</td>
<td>Pal</td>
<td>1</td>
</tr>
<tr>
<td>Potri.016G126300.1</td>
<td>AT3G51740.1</td>
<td>Leucine-rich repeat receptor kinase/IMK2</td>
<td>Mem</td>
<td>Signal Transduction</td>
<td>1.36</td>
<td>20.3 (12)</td>
<td>Pal</td>
<td>1</td>
</tr>
<tr>
<td>Potri.007G065000.1</td>
<td>AT4G354700.1</td>
<td>Plant intracellular Ras-group related LRR 4</td>
<td>Mem</td>
<td>Signal Transduction</td>
<td>1.63</td>
<td>42 (16)</td>
<td>Pal</td>
<td>0</td>
</tr>
<tr>
<td>Potri.005G242000.1</td>
<td>AT4G35020.1</td>
<td>RAC-like 3/ROP6</td>
<td>Mem</td>
<td>Signal Transduction</td>
<td>1.36</td>
<td>15.2 (2)</td>
<td>Pal</td>
<td>0</td>
</tr>
<tr>
<td>Potri.015G073000.1</td>
<td>AT3G48040.1</td>
<td>RAC-like 8/ROP10</td>
<td>Mem</td>
<td>Signal Transduction</td>
<td>2.96</td>
<td>19.9 (2)</td>
<td>Pal</td>
<td>0</td>
</tr>
<tr>
<td>Potri.012G044600.1</td>
<td>AT1G48480.1</td>
<td>Receptor-like protein kinase 1</td>
<td>Mem</td>
<td>Signal Transduction</td>
<td>2.06</td>
<td>27.9 (13)</td>
<td>Pal</td>
<td>1</td>
</tr>
<tr>
<td>Potri.014G113500.1</td>
<td>AT3G62150.1</td>
<td>ABC transporter/ MDR17</td>
<td>Mem</td>
<td>Transport</td>
<td>1.91</td>
<td>22.8 (19)</td>
<td>Pal</td>
<td>12</td>
</tr>
<tr>
<td>Potri.002G187500.1</td>
<td>AT2G47000.1</td>
<td>ABC transporter/ MDR4</td>
<td>Mem</td>
<td>Transport</td>
<td>2.5</td>
<td>37.7 (28)</td>
<td>Pal</td>
<td>12</td>
</tr>
<tr>
<td>Potri.014G113000.1</td>
<td>AT2G47000.1</td>
<td>ABC transporter/ MDR4</td>
<td>Mem</td>
<td>Transport</td>
<td>1.8</td>
<td>24.9 (20)</td>
<td>Pal</td>
<td>12</td>
</tr>
<tr>
<td>Potri.001G189500.1</td>
<td>AT1G59870.1</td>
<td>ABC transporter/ PDR8</td>
<td>Mem</td>
<td>Transport</td>
<td>1.7</td>
<td>19.3 (21)</td>
<td>Pal</td>
<td>13</td>
</tr>
<tr>
<td>Potri.008G082100.1</td>
<td>AT3G12740.1</td>
<td>ALA-interacting subunit 1, ALIS1</td>
<td>Mem</td>
<td>Transport</td>
<td>2.3</td>
<td>28.3 (8)</td>
<td>Pal</td>
<td>2</td>
</tr>
<tr>
<td>Potri.012G131200.1</td>
<td>AT4G21120.1</td>
<td>Amino acid transporter 1</td>
<td>Mem</td>
<td>Transport</td>
<td>1.72</td>
<td>14.9 (6)</td>
<td>Pal</td>
<td>14</td>
</tr>
<tr>
<td>Potri.010G063500.1</td>
<td>AT4G13510.1</td>
<td>Ammonium transporter 1</td>
<td>Mem</td>
<td>Transport</td>
<td>1.2</td>
<td>15.4 (14)</td>
<td>Pal</td>
<td>10</td>
</tr>
<tr>
<td>Potri.006G072900.1</td>
<td>AT4G29900.1</td>
<td>Ca2+-ATPase 10</td>
<td>Mem</td>
<td>Transport</td>
<td>1.68</td>
<td>35.2 (24)</td>
<td>Pal</td>
<td>12</td>
</tr>
<tr>
<td>Potri.006G083000.1</td>
<td>AT3G50360.1</td>
<td>Centrin 2</td>
<td>Mem</td>
<td>Transport</td>
<td>1.44</td>
<td>46.7 (5)</td>
<td>Pal</td>
<td>0</td>
</tr>
<tr>
<td>Potri.006G083000.1</td>
<td>AT4G16480.1</td>
<td>Inositol transporter 4</td>
<td>Mem</td>
<td>Transport</td>
<td>1.7</td>
<td>5.7 (3)</td>
<td>Pal</td>
<td>12</td>
</tr>
<tr>
<td>Potri.001G335300.1</td>
<td>AT5G40780.1</td>
<td>Lysine histidine transporter 1</td>
<td>Mem</td>
<td>Transport</td>
<td>2.41</td>
<td>32.8 (12)</td>
<td>Pal</td>
<td>9</td>
</tr>
<tr>
<td>Potri.002G234700.1</td>
<td>AT1G14780.1</td>
<td>MAC/Perforin domain-containing protein</td>
<td>Unk</td>
<td>Transport</td>
<td>1.36</td>
<td>21.5 (8)</td>
<td>Pal</td>
<td>0</td>
</tr>
<tr>
<td>Potri.002G095900.1</td>
<td>AT1G34580.1</td>
<td>Major facilitator superfamily protein</td>
<td>Mem</td>
<td>Transport</td>
<td>2.67</td>
<td>10.8 (5)</td>
<td>Pal</td>
<td>11</td>
</tr>
<tr>
<td>Potri.015G081300.1</td>
<td>AT1G12940.1</td>
<td>Nitrate transporter 2.5</td>
<td>Mem</td>
<td>Transport</td>
<td>1.91</td>
<td>15.6 (6)</td>
<td>Pal</td>
<td>12</td>
</tr>
<tr>
<td>Potri.015G081300.1</td>
<td>AT5G02020.1</td>
<td>Nitrate transporter 3.1</td>
<td>Mem</td>
<td>Transport</td>
<td>1.94</td>
<td>29.8 (5)</td>
<td>Pal</td>
<td>2</td>
</tr>
<tr>
<td>Potri.009G079500.1</td>
<td>AT5G64410.1</td>
<td>Oligopeptide transporter 4</td>
<td>Mem</td>
<td>Transport</td>
<td>3.45</td>
<td>15.8 (7)</td>
<td>Pal</td>
<td>15</td>
</tr>
<tr>
<td>Potri.012G091700.1</td>
<td>AT4G27730.1</td>
<td>Oligopeptide transporter 6</td>
<td>Mem</td>
<td>Transport</td>
<td>2.85</td>
<td>10.4 (5)</td>
<td>Pal</td>
<td>15</td>
</tr>
<tr>
<td>Potri.006G320500.1</td>
<td>AT2G38940.1</td>
<td>Phosphate transporter 2</td>
<td>Mem</td>
<td>Transport</td>
<td>4.5</td>
<td>27.3 (10)</td>
<td>Pal</td>
<td>12</td>
</tr>
<tr>
<td>Potri.008G065600.1</td>
<td>AT4G00430.1</td>
<td>Plasma membrane intrinsic protein 1;4</td>
<td>Mem</td>
<td>Transport</td>
<td>2.54</td>
<td>37.3 (5)</td>
<td>Pal</td>
<td>6</td>
</tr>
<tr>
<td>Potri.010G191900.1</td>
<td>AT4G00430.1</td>
<td>Plasma membrane intrinsic protein 1;4</td>
<td>Mem</td>
<td>Transport</td>
<td>1.55</td>
<td>33.8 (4)</td>
<td>Pal</td>
<td>6</td>
</tr>
<tr>
<td>Potri.003G128600.1</td>
<td>AT4G00430.1</td>
<td>Plasma membrane intrinsic protein 1;4</td>
<td>Mem</td>
<td>Transport</td>
<td>2.96</td>
<td>14.6 (3)</td>
<td>Pal</td>
<td>7</td>
</tr>
<tr>
<td>Potri.004G176300.1</td>
<td>AT4G35100.1</td>
<td>Plasma membrane intrinsic protein 2;7</td>
<td>Mem</td>
<td>Transport</td>
<td>4.00</td>
<td>14.3 (3)</td>
<td>Pal</td>
<td>6</td>
</tr>
<tr>
<td>Potri.018G090300.1</td>
<td>AT2G18960.1</td>
<td>P-type H+-ATPase 1</td>
<td>Mem</td>
<td>Transport</td>
<td>2.5</td>
<td>43.4 (26)</td>
<td>Pal</td>
<td>10</td>
</tr>
<tr>
<td>Accessory Code</td>
<td>Accessory ID</td>
<td>Description</td>
<td>Membrane Location</td>
<td>Function</td>
<td>Log2Ratio</td>
<td>Fold Change</td>
<td>Significance</td>
<td>Palmitoylation</td>
</tr>
<tr>
<td>---------------</td>
<td>--------------</td>
<td>-------------</td>
<td>-------------------</td>
<td>----------</td>
<td>------------</td>
<td>-------------</td>
<td>--------------</td>
<td>---------------</td>
</tr>
<tr>
<td>Potri.012G071600.1</td>
<td>AT5G62670.1</td>
<td>P-type H+-ATPase 11</td>
<td>Mem</td>
<td>Transport</td>
<td>2.31</td>
<td>28.6 (17)</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Potri.015G066000.2</td>
<td>AT5G62670.1</td>
<td>P-type H+-ATPase 11</td>
<td>Mem</td>
<td>Transport</td>
<td>1.85</td>
<td>15.2 (9)</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Potri.006G275000.1</td>
<td>AT2G24520.1</td>
<td>P-type H+-ATPase 5</td>
<td>Mem</td>
<td>Transport</td>
<td>2.19</td>
<td>20.6 (11)</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Potri.018G006000.1</td>
<td>AT2G24520.1</td>
<td>P-type H+-ATPase 5</td>
<td>Mem</td>
<td>Transport</td>
<td>1.54</td>
<td>23.7 (16)</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Potri.012G058000.1</td>
<td>AT1G68710.1</td>
<td>Secretory carrier membrane protein 3</td>
<td>Mem</td>
<td>Transport</td>
<td>1.63</td>
<td>21.7 (4)</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Potri.004G033600.1</td>
<td>AT1G11260.1</td>
<td>Sugar transporter 1</td>
<td>Mem</td>
<td>Transport</td>
<td>1.41</td>
<td>43.5 (16)</td>
<td>Pal 12</td>
<td></td>
</tr>
<tr>
<td>Potri.001G253800.1</td>
<td>AT1G77210.1</td>
<td>Sugar transporter 14</td>
<td>Mem</td>
<td>Transport</td>
<td>1.88</td>
<td>44.8 (16)</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Potri.016G088200.1</td>
<td>AT3G09740.1</td>
<td>Syntaxin 71</td>
<td>Mem</td>
<td>Transport</td>
<td>1.65</td>
<td>83.4 (16)</td>
<td>Pal 1</td>
<td></td>
</tr>
<tr>
<td>Potri.003G036800.1</td>
<td>AT5G53550.1</td>
<td>Yellow Stripe like 3</td>
<td>Mem</td>
<td>Transport</td>
<td>2.46</td>
<td>38.6 (19)</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Potri.001G366100.1</td>
<td>AT1G55910.1</td>
<td>Zinc transporter 11</td>
<td>Mem</td>
<td>Transport</td>
<td>3.5</td>
<td>22.5 (5)</td>
<td>Pal 8</td>
<td></td>
</tr>
<tr>
<td>Potri.006G258900.1</td>
<td>AT5G62890.1</td>
<td>Band 7-containing membrane protein</td>
<td>Mem</td>
<td>Unclassified</td>
<td>2.45</td>
<td>71.5 (25)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Potri.005G234900.1</td>
<td>AT1G05150.1</td>
<td>Calcium-binding tetratricopeptide protein</td>
<td>Mem</td>
<td>Unclassified</td>
<td>1.27</td>
<td>18.7 (10)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Potri.001G358300.1</td>
<td>AT1G65730.1</td>
<td>Yellow Stripe like 7</td>
<td>Mem</td>
<td>Transport</td>
<td>1.63</td>
<td>7.4 (4)</td>
<td>Pal 14</td>
<td></td>
</tr>
<tr>
<td>Potri.001G366100.1</td>
<td>AT1G55910.1</td>
<td>Zinc transporter 11</td>
<td>Mem</td>
<td>Transport</td>
<td>3.5</td>
<td>22.5 (5)</td>
<td>Pal 8</td>
<td></td>
</tr>
<tr>
<td>Potri.006G258900.1</td>
<td>AT5G25260.1</td>
<td>Band 7-containing membrane protein</td>
<td>Mem</td>
<td>Unclassified</td>
<td>1.61</td>
<td>71.5 (25)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Potri.005G234900.1</td>
<td>AT1G05150.1</td>
<td>Calcium-binding tetratricopeptide protein</td>
<td>Mem</td>
<td>Unclassified</td>
<td>1.27</td>
<td>18.7 (10)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Potri.001G358300.1</td>
<td>AT1G30290.1</td>
<td>Early-responsive to dehydration 4</td>
<td>Mem</td>
<td>Unclassified</td>
<td>1.83</td>
<td>14 (8)</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Potri.019G031500.1</td>
<td>AT2G39050.1</td>
<td>Euonymus lectin S3</td>
<td>Unk</td>
<td>Unclassified</td>
<td>1.61</td>
<td>29.1 (6)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Potri.017G142800.1</td>
<td>AT5G23980.1</td>
<td>Ferric reduction oxidase 4</td>
<td>Mem</td>
<td>Unclassified</td>
<td>1.33</td>
<td>14.5 (7)</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Potri.003G112700.1</td>
<td>AT4G12420.1</td>
<td>SKU5 Cupredoxin superfamily protein</td>
<td>Mem</td>
<td>Unclassified</td>
<td>1.39</td>
<td>23.6 (9)</td>
<td>Pal 1</td>
<td></td>
</tr>
<tr>
<td>Potri.009G015100.1</td>
<td>AT3G45600.1</td>
<td>Tetracitin 3</td>
<td>Mem</td>
<td>Unclassified</td>
<td>1.75</td>
<td>27.8 (7)</td>
<td>Pal 4</td>
<td></td>
</tr>
<tr>
<td>Potri.016G068000.1</td>
<td>AT3G44150.1</td>
<td>Unknown protein</td>
<td>Mem</td>
<td>Unclassified</td>
<td>2.33</td>
<td>36.5 (7)</td>
<td>Pal 1</td>
<td></td>
</tr>
</tbody>
</table>
FIG. 1. Experimental set-up and strategy used for data analysis. PMF, plasma membrane fraction; DRM, detergent-resistant microdomains; TE, Triton extract; iTRAQ, isobaric tags for relative and absolute quantitation; SCX, strong cation-exchange chromatography.
FIG. 2. Qualitative proteomic analysis of PMF (plasma membrane fraction), DRM (detergent-resistant microdomains) and TE (Triton extract). The Venn diagram represents the number of proteins identified from SDS-PAGE of PMF, DRM and TE. In total, 917, 267 and 342 proteins were identified by MS analysis of PMF, DRM and TE, respectively.
FIG. 3. Predicted cellular localization (A, B) and functional classification (C, D) of proteins identified after SDS-PAGE analysis of DRM and PMF. (A and C) DRM (detergent-resistant microdomains); (B and D) PMF (plasma membrane fraction). Gene ontology information was retrieved from TAIR (www.arabidopsis.org).
FIG. 4. SDS-PAGE analysis of proteins present in DRM (detergent-resistant microdomains), TE (Triton extract) and PMF (plasma membrane fraction). A total of 20 µg protein was loaded in each lane. Each lane of the Coomassie-blue-stained gel was cut into 48 bands as shown on the left side of the picture. Arrowheads point to protein bands of a higher intensity in DRM compared to TE and PMF.

FIG. 5. Functional classification of the 80 proteins enriched in the poplar DRM based on gene ontology prediction. Gene ontology information was retrieved from TAIR (www.arabidopsis.org).
FIG. 6. Classification of DRM-enriched (green bars) and total plasma membrane proteins (blue bars) based on their number of transmembrane domains (TMD) (A), average number of amino acids in TMD (B), molecular mass (C) and isoelectric point (D).
FIG. 7. **Integrated hypothetical model representing the different proteins enriched in the poplar DRM and the biological processes in which they are involved.** Proteins whose names are highlighted in blue were shown to be enriched in plasma membrane DRM (grey patches) together with other proteins listed in Table I. All proteins enriched in DRM are markers of signal transduction, molecular transport and callose biosynthesis and known to be involved in biotic and/or abiotic stress. Vesicles carrying proteins involved in endocytosis and exocytosis and cell wall biosynthetic enzymes (callose synthases or GSL) are represented by green circles. The
represented cascade of events leads to the release of calcium on the cytoplasmic side of the plasma membrane, which is required for the activation of callose synthase as a final response to stress (80). The enrichment of callose synthase in DRM suggests a preferential deposition of callose plugs at the surface of plasma membrane microdomains, possibly in specialized plasmodesmatal structures and/or other sites of the plasma membrane. DAG, diacylglycerol; DGK, diacylglycerol kinase; ER, endoplasmic reticulum; GSL, glucan synthase-like/callose synthase; IP3, inositol-3-phosphate; PIP, phosphatidyl-inositol-phosphate; PL, phospholipid; PLC, phosphatidylinositol phospholipase C; PLD, phospholipase D; PM, plasma membrane; SCAMPS, secretory carrier membrane proteins; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor.