A versatile method for deciphering plant membrane proteomes

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Abstract
Proteomics is a very powerful approach to link the information contained in sequenced genomes, like that of Arabidopsis, to the functional knowledge provided by studies of plant cell compartments. This article summarizes the different steps of a versatile strategy that has been developed to decipher plant membrane proteomes. Initiated with envelope membranes from spinach chloroplasts, this strategy has been adapted to thylakoids, and further extended to a series of membranes from the model plant Arabidopsis: chloroplast envelope membranes, plasma membrane, and mitochondrial membranes. The first step is the preparation of highly purified membrane fractions from plant tissues. The second step in the strategy is the fractionation of membrane proteins on the basis of their physico-chemical properties. Chloroform/methanol extraction and washing of membranes with NaOH, NaCl or any other agent led to the simplification of the protein content of the fraction to be analysed. The next step is the genuine proteomic step, i.e. the separation of proteins by 1D-gel electrophoresis followed by in-gel proteolytic digestion of the polypeptides, analysis of the proteolytic peptides using mass spectrometry, and protein identification by searching through databases. The last step is the validation of the procedure by checking the subcellular location. The results obtained by using this strategy demonstrate that a combination of different proteomics approaches, together with bioinformatics, indeed provide a better understanding of the biochemical machinery of the different plant membranes at the molecular level.

Key words: Arabidopsis, membrane proteins, plant, proteomics, subcellular localization.

Introduction
Membrane proteins play a key role because of their position at the interface between cells or between cell compartments. For instance, the main function of the plasma membrane is to regulate the exchanges of information, ions, and metabolites between the cell and its surrounding environment. The plant plasma membrane carries additional functions, such as cell wall biosynthesis and responses to biotic and abiotic factors. Another example is that of the chloroplast envelope membranes (Douce and Joyard, 1990): the functional integration of chloroplasts within the plant cell requires a series of unique transporters restricted to the chloroplast envelope membranes such as metabolite transporters, ion channels, pumps, permeases, pore proteins, etc.

Identification of the genes encoding membrane proteins is a key issue. Only a minority of integral membrane proteins have been confirmed as proteins and most of them came from in silico analyses once the genome of the model plant Arabidopsis thaliana had been completed (Arabidopsis Genome Initiative, 2000). Several databases (such as AMPL, Aramemnon, etc.) focus on plant membrane
proteins (see review by Schwacke et al., 2004). AMPL (Arabidopsis Membrane Protein Library, http://www.cbs.umn.edu/Arabidopsis/), was established by clustering the predicted membrane proteins based on sequence and sorted into families of known or predicted function or unknown function (Ward, 2001). It was recently extended to rice membrane proteins (http://www.cbs.umn.edu/rice/). Aramemnon, a specialized database for Arabidopsis integral membrane proteins averaging the predictions from different publicly available programs, identified approximately 6500 proteins displaying at least one transmembrane domain of the 25 500 predicted protein sequences (http://aramemnon.botanik.uni-koeln.de). Some 1800 of these proteins contain at least four transmembrane domains (Schwacke et al., 2003).

Proteomics is one of the possible analytical strategies for the comprehensive identification of membrane proteins (Barbier-Brygoo et al., 2001). However, membrane proteomics has been a major challenge, mostly because membrane proteins are rather more difficult to analyse than soluble proteins. Obviously, integral membrane proteins summarize all the major problems that can occur when performing proteome analyses. Firstly, membrane proteins are often minor constituents and the dynamic of their expression in the cell is difficult to approach. Rare proteins are present at levels in the order of 10 to 100 molecules per cell, whereas the most abundant proteins are present at levels between 10⁵–10⁷ molecules per cell. This extremely wide range of expression clearly prevents any exhaustive visualization and characterization of minor proteins in total cell extracts. This means that low-abundance proteins, including regulatory proteins and rare membrane proteins, are out of the scope of most proteomic techniques unless specific strategies are developed.

Secondly, the chemical heterogeneity of proteins, which is directly linked to the diversity of their functions, is another major problem for proteomics and especially when membrane proteins are considered. In general, proteins cover a range of isoelectric points (pI) from 3 to more than 12 and have a range of Mᵋ from fewer than 5000 to more than 300 000 Daltons. For instance, Ferro et al. (2002) demonstrated that a large proportion of the chloroplast envelope membrane proteins exhibit an alkaline isoelectric point (above 8.5). Despite new developments, this heterogeneity is a major challenge for 2D-gel electrophoretic procedures based on pI and Mᵋ for separation. Furthermore, membranes can be considered as being made of a continuum of hydrophilic to hydrophobic proteins, i.e. from water-soluble proteins (present in compartments like the cytosol or the mitochondrial matrix), to the most hydrophobic core of the membrane made of proteins containing several transmembrane domains embedded within the lipid phase of the membrane. In contrast to soluble proteins, highly hydrophobic membrane proteins largely escape proteomic analyses based on 2D-gel technology because of their low abundance and their limited solubility in water (Santoni et al., 2000).

Despite all these limitations, a large set of data is now available for some of the major plant membrane proteomes (for reviews see Rolland et al., 2003; Whitelegge, 2003; Ephritikhine et al., 2004; Eubel et al., 2004; van Wijk, 2004). For instance, in the case of Arabidopsis plasma membrane, whereas the majority of proteins identified in the pioneering studies were peripheral proteins (Santoni et al., 1998, 1999), recent studies have led to the identification of a reasonable number of intrinsic membrane proteins in this system (Alexandersson et al., 2004; Marmagne et al., 2004). The most comprehensive subcellular proteome database, including membrane proteins, concerns the chloroplast and its constituents (PPDB; http://ppdb.tc.cornell.edu/; Peltier et al., 2004; more than 700 plastid membrane (envelope and thylakoid) proteins have been identified in Arabidopsis (Ferro et al., 2000, 2002, 2003; Froehlich et al., 2003; Friso et al., 2004). In the case of plant mitochondria (Millar et al., 2001; Millar and Heazlewood, 2003; Brugiére et al., 2004; Heazlewood et al., 2004), alternatives to 2D-electrophoresis (especially blue-native PAGE separations) were used to identify membrane proteins (Kruft et al., 2001; Eubel et al., 2004).

This article summarizes the different steps of a versatile strategy developed to decipher plant membrane proteomes. Initiated with envelope membranes from spinach chloroplasts (Seigneurin-Berny et al., 1999), this strategy has been adapted to thylakoids (Ferro et al., 2000), and further extended to a series of membranes from the model plant Arabidopsis: chloroplast envelope membranes (Ferro et al., 2003), plasma membrane (Marmagne et al., 2004), and mitochondrial membranes (Brugiére et al., 2004). The first step is the preparation of highly purified membrane fractions from plant tissues. Despite the difficulty in handling Arabidopsis for efficient cell fractionation, this model plant was used to take advantage of the genomic knowledge provided by the complete sequencing of the genome (Arabidopsis Genome Initiative, 2000). The second step in this strategy is the fractionation of membrane proteins on the basis of their physico-chemical properties. Chloroform/methanol extraction and washing of membranes with NaOH, NaCl or any other agent, led to the simplification of the protein content of the fraction to be analysed. The next step is the genuine proteomic step, i.e. the separation of proteins by 1D-gel electrophoresis, followed by the in-gel proteolytic digestion of the polypeptides, analysis of the proteolytic peptides by using mass spectrometry, and protein identification by searching through databases. The last step is the validation of the procedure by checking the subcellular location. This is essential since (i) many proteins are unknown and (ii) targeting sequences to a given membrane system are not always obvious.
Preparation and characterization of highly purified membrane systems from Arabidopsis

In general, a biochemical approach to address a given biological question is developed on the most suitable biological material. Since (i) large scale and high throughput is essential for proteomics, and (ii) the sequence of very few proteins is known and from only a small number of species, a key point is to analyse a model system for which a large amount of genomic data (DNA sequences, ESTs) are available. Therefore plants such as Arabidopsis or rice are more appropriate organisms for proteomics than spinach, despite the poor interest in Arabidopsis for accurate subcellular fractionation. However, and despite the tremendous progress made in proteomics, the efficiency of this technology strongly relies on the quality of the biological sample analysed. This points to sample preparation and handling as the most critical step in proteomics. Again, Arabidopsis is probably not the best plant model to achieve a high level of membrane purification. As an example of the whole strategy that should be used for membrane proteomics, the preparation and characterization of envelope membranes from Arabidopsis chloroplasts is described below. This description illustrates the key steps in membrane purification and the questions to be addressed prior to membrane proteome analysis.

Purification of envelope membranes from Arabidopsis chloroplasts

Envelope membranes represent only a minor proportion of the total chloroplast proteins (about 1–2%), therefore this fraction can be prepared in a reasonably pure state from chloroplasts and is devoid of contaminants such as mitochondria, endoplasmic reticulum, etc. This is a really critical stage in envelope membrane preparation. Methods to prepare highly purified chloroplasts have been developed, mostly using spinach, a plant of choice for such experiments (Douce and Joyard, 1982). Successful adaptation of spinach procedures to the purification of Arabidopsis chloroplasts was recently achieved (Ferro et al., 2003). This process consists in (i) the homogenization of leaf tissue using a Waring blender in buffered medium and an osmoticum, (ii) the recovery of a crude preparation of chloroplasts using differential centrifugations to eliminate most of the possible contaminants deriving from the cytosol, the vacuole, and any broken organelle, (iii) the purification of chloroplasts using Percoll gradients to eliminate membranes from broken chloroplasts (mostly thylakoids) and from other organelles. Indeed, Percoll-purified chloroplast preparations are devoid of contamination by extra-plastidial marker enzymes (like glycolate oxidase, catalase, fumarase, nitrate reductase) and non-chloroplastic membrane lipids (phosphatidylethanolamine and cardiolipin) (for reviews see Douce and Joyard, 1982, 1990). The high purity of Percoll-purified chloroplasts was more recently confirmed through the proteomic analysis of their envelope membranes (Ferro et al., 2002). No protein known to be localized outside the chloroplast was identified from a total of 54 in spinach envelope membranes, whereas six out of 112 of the identified Arabidopsis proteins may correspond to non-plastid proteins (Ferro et al., 2003). Among them, one protein appeared to correspond to a previously characterized major plasma membrane component, whereas four proteins may indicate contamination by major tonoplast proteins and one from glyoxysomes. Considering the high sensitivity of present mass spectrometers it was not surprising to detect a few peptides deriving from these few extra-plastidial protein contaminants, which are major proteins in their respective subcellular compartment. However, since the subcellular localization of some of these proteins remains to be unambiguously determined, the contamination of the Percoll-purified Arabidopsis chloroplasts may be overestimated. The unique plastid localization of a phosphate transporter (Ferro et al., 2002), previously suspected to be associated with the plasma membrane (Daram et al., 1999) was demonstrated. It is also important to note that none of the 112 identified Arabidopsis proteins appears to derive from mitochondria, a classical contaminant of plastid preparations (Douce and Joyard, 1982).

The method used to prepare envelope membranes from purified intact spinach chloroplasts, and based on chloroplast lysis in a hypotonic medium followed by centrifugation on sucrose gradients (Douce and Joyard, 1982), cannot be used for Arabidopsis in a straightforward manner. In this case, mechanical processes or freeze/thaw cycles are required to break the envelope membranes and release the thylakoids and the stroma into the medium. As a consequence, cross-contamination of the envelope and thylakoid membranes is higher than when a simple osmotic shock is sufficient. In both cases, such preparations contain the whole chloroplast envelope system, i.e. the outer and the inner envelope membranes. [Procedures have been developed to separate the outer and the inner envelope membranes (Cline et al., 1981; Block et al., 1983), but strong limitation to the use of such membrane preparations for proteomics still exist such as (i) the cross contamination of outer membrane preparation by the inner membrane, and vice versa, (ii) the low yield of membrane preparation, and (iii) the fact that the methods used to separate the two membranes are generally most efficient only with spinach and pea. In addition, most outer envelope membrane proteins are devoid of chloroplast targeting sequences and therefore cannot be easily distinguished from cytosolic proteins.]

However, even for Arabidopsis, the use of Percoll-purified chloroplasts is very efficient at limiting the contamination of envelope membranes by extraplastidial membranes. At this stage, the major possible contaminants of envelope preparations are soluble stroma proteins and
small pieces of thylakoid membranes. Such cross contaminations have been extensively analysed. Electrophoretic analyses of chloroplast subfractions show that their polypeptide patterns are strikingly different. RuBisCo, the major stroma protein, is detected in other subplastidial fractions, but only at a very low level. LHCPs, the major thylakoid proteins, were highly enriched in the thylakoid fraction, but hardly visible in other subplastidial fractions. Being the most likely source of membrane contamination of the purified envelope fraction, thylakoid cross-contamination needs to be precisely assessed. The yellow colour of purified envelope vesicles first indicates that this membrane system contain almost no chlorophyll and therefore very few contaminating thylakoids. Indeed, by western blot analyses using antibodies raised against LHCP, Ferro et al. (2003) demonstrated that several independent Arabidopsis envelope preparations appeared to contain between 1% and 3% thylakoid proteins.

Purifying Arabidopsis membranes for proteomics

The description of an efficient strategy to prepare highly purified envelope membranes from Arabidopsis chloroplasts (Fig. 1A) raises several general points. Firstly, the best biological material is needed to prepare the membrane system of interest. The choice of leaf tissue for preparing chloroplasts seems obvious, but this was not the case for mitochondria since their purification from Arabidopsis leaves is very difficult to achieve. They are generally contaminated and inactive when extracted from leaves (or other plant tissues), whereas reasonably pure and active mitochondria can more easily be extracted from Arabidopsis cell suspensions (Brugière et al., 2004). The same is true for plasma membrane. In such cases, the use of cell cultures seems more obvious.

In all cases, the best methods to obtain highly purified plant membranes rely on (i) tissue and cell breakage using mechanical processes in a buffered medium (to limit proteolysis attack due to acidity of the vacuolar content) containing an osmoticum (to maintain the integrity of the organelles), (ii) a series of differential centrifugations, to enrich the targeted intact organelle and to eliminate as much as possible contaminants from other cell compartments, (iii) purification of the targeted organelle (e.g. plastids or mitochondria) on density gradients or by two-phase partitioning in aqueous polymers (e.g. plasma membrane).

Despite the fact that the yield is low (few %), as previously discussed by Ephritikhine et al. (2004), purity should be the first priority before yield and it is essential to start from large amounts of tissues (even for small plants like Arabidopsis). Purity of the fractions of interest should be monitored, for instance by measuring enzymatic activities or by immunodetection for selected enzymatic markers, to estimate the contamination deriving from other subcellular compartments (Ephritikhine et al., 2004).

Fig. 1. (A) Purification and characterization of plant membranes. Highly purified envelope membranes were prepared from Arabidopsis chloroplasts from plantlets, whereas membranes from mitochondria and plasma membrane were prepared from cell cultures. The different membrane fractions are then characterized to determine their level of purity (Ferro et al., 2003; Brugière et al., 2003; Marmagne et al., 2004). (B) Combination of extraction procedures. The use of complementary extraction methods (i.e. chloroform/methanol extraction, alkaline and saline treatments) provided a rather broad view of Arabidopsis membrane proteomes (Ferro et al., 2003; Brugière et al., 2003; Marmagne et al., 2004). Organic solvent treatment of plant membranes provides a new insight on the hydrophobic core of Arabidopsis membrane systems. Alkaline and saline treatments of purified membrane fractions result in the extraction of different sets of proteins: Na2CO3 or NaCl extract proteins that are rather weakly associated with the membrane whereas NaOH removes proteins that are more tightly associated.
[For instance, Marmagne et al. (2004) have estimated the activity of three marker enzymes in plasma membrane preparation compared with crude microsomal fraction. The ratio between the activity of the nitrate-sensitive ATPase in the plasma membrane fraction and the activity of the microsomal fraction was 0.9±0.1. The ratio between the activity of the vanadate-sensitive ATPase in the plasma membrane fraction and the activity of the microsomal fraction was 3.7±0.3. The ratio of the vanadate-sensitive ATPase activity in the plasma membrane to the total ATPase activity in the same fraction was 91.6. This suggests that the plasma membrane contamination by tonoplast and other membranes displaying ATPase activities (endoplasmic reticulum, mitochondria, chloroplasts) was at most 8%, a likely over-estimation considering the relative lack of specificity of the inhibitors used in the enzymatic assays. The ratio between the activity of cytochrome c oxidase in the plasma membrane fraction and the activity of the microsomal fraction was \(2.9 \times 10^{-3} \pm 2 \times 10^{-3}\), corresponding to an extremely low level of mitochondrial contamination in the plasma membrane fraction.]

Proteome analyses further provide quality control of the fractions: it was observed that envelope preparations were mostly contaminated with stroma- and thylakoid-deriving proteins while plasma membrane contaminants were mainly deriving from the cytosol.

Percoll-purified plastids or mitochondria contain very few protein contaminants from the cytosol (Ferro et al., 2003; Brugières et al., 2004) and the use of Percoll gradients is much more reliable than that of sucrose gradients for organelle purification (Ephritikhine et al., 2004). For instance, only 2% of the proteins identified by proteomics in Arabidopsis mitochondria preparations were actually localized in other compartments (Brugières et al., 2004). Concerning plasma membrane, the two-phase partitioning in aqueous polymers (a mixture of dextran and polyethylene glycol) is the most suitable technique, as the PEG upper phase is highly enriched in plasma membrane while all the endomembranes are concentrated in the lower phase. This is an efficient procedure since plasma membranes prepared according to this procedure contain no or very little proteins from other compartments. [However, a key point, when membranes are purified by two-phase partition, is to eliminate the PEG adsorbed at the vesicle surface. This has been extensively described by Marmagne et al. (2004, 2006).] For instance, the proteomic analysis of Arabidopsis plasma membranes (Marmagne et al., 2004) did not reveal the presence of peptides deriving from the RuBisCo, the major chloroplast protein, of the chloroplast envelope phosphate/triose-phosphate translocator, or from any of the constituents of the four dominant protein complexes in mitochondria (complex I, HSP60 complex, F_{0}F_{1}-ATP synthase or cytochrome c reductase).

Membrane fractionation, a tool to reveal membrane protein diversity

The next step in membrane proteomics strategy is to reduce the complexity of the membrane fractions to be analysed to get a broader view of the system. The objective is to bring low-abundance and hydrophobic proteins into view for proteomic analysis. 2D-gel electrophoresis combined with the use of appropriate detergents can be very efficient for analysing some plasma membrane proteins like aquaporins (Santoni et al., 2003). Unfortunately, detergents cannot be used for membrane systems like the chloroplast envelope membranes that contain a high lipid to protein ratio (Douce and Joyard, 1990), despite the alternative procedures developed to widen the scope of useful detergents for the solubilization of membrane proteins in proteomics (Rabilloud et al., 1997; Chevallet et al., 1998; Luche et al., 2003). Several other methods, based on the differential extraction of proteins by organic solvents, salts, detergents, etc. have been developed to analyse membrane proteins from the less to the more hydrophobic ones (Ferro et al., 2003; Brugières et al., 2004; Friso et al., 2004; Marmagne et al., 2004; Peltier et al., 2004) using 1D-SDS-PAGE analyses.

Differential solubilization of membrane proteins in chloroform/methanol mixtures

Seigneurin-Berny et al. (1999) developed a simple and efficient strategy specifically to extract the most hydrophobic proteins present in chloroplast envelope membranes (Fig. 1B). This method was based on the differential solubilization of membrane proteins in chloroform/methanol mixtures, which allows extracting and concentrating the most hydrophobic membrane proteins. The propensity of membrane proteins to partition in chloroform/methanol mixtures was directly correlated with their hydrophobicity, such as the Res/TM ratio (number of amino acid residues/number of putative transmembrane regions). Furthermore, chloroform/methanol extraction of membrane proteins (i) eliminates peripheral proteins as well as soluble contaminants from membrane fractions and (ii) limits protein pattern complexity. Indeed, when compared to the generally used differential solubilization in detergents, chloroform/methanol extractions seems to be the best possible compromise to combine enrichment of highly hydrophobic proteins and extensive elimination of the hydrophilic ones (Ferro et al., 2000).

Indeed, about 80% of the proteins identified in chloroform/methanol extracts from chloroplast envelope membranes (Ferro et al., 2002, 2003) contain at least one predicted transmembrane domain. Those without any predicted transmembrane domain could be classified into three categories: (i) genuine integral membrane proteins that contain amphipathic β-strands spanning the membrane (e.g. OEP21; Bolter et al., 1999); (ii) stroma and peripheral envelope...
proteins; and (iii) genuine envelope proteins that are extracted in chloroform/methanol solutions because of strong interactions with lipids (MGDG synthase; Miège et al., 1999). Some of the 40 proteins found to have at least four predicted α-helical transmembrane domains are well-characterized envelope transporters, such as the envelope phosphate/triose phosphate translocator and dicarboxylate transporters. However, most of these proteins are either unknown proteins or proteins with putative functions.

In addition to studies of plastid envelope membranes (Seigneurin-Berny et al., 1999; Ferro et al., 2002, 2003), this method was successfully applied to other plant membrane systems such as thylakoid membranes (Ferro et al., 2000; Friso et al., 2004), mitochondrial membranes (Millar and Heazlewood, 2003; Brugière et al., 2004) and plasma membrane (Marmagne et al., 2004).

The proportion of chloroform/methanol to be used should be carefully determined for each type of membrane to be analysed: a careful analysis of the polypeptide profiles obtained by 1D-gel electrophoresis is the best way to determine the adequate proportion of chloroform to methanol (Ferro et al., 2000). [The volume ratio between chloroform and methanol for an optimal extraction can be determined by comparing the polypeptide profile of the organic phase soluble proteins prepared as follows: membranes (5 mg proteins in 1 ml storage buffer) are divided in 10 fractions of 0.1 ml (in 1.5 ml Eppendorf tubes). The membrane fraction is then slowly diluted by addition of 0.9 ml of cold chloroform/methanol solutions (0:9, 1:8, 2:7, 3:6, 4:5, 5:4, 6:3, 7:2, 8:1 and 9:0, v/v). In general, the total volume of the mixture is 1 ml. If necessary, this can be increased to a much higher value when more membrane material is available. This method was extensively described by Ferro et al. (2000).]

Ephritikhine et al. (2004) identified three major limits when using chloroform/methanol to solubilize membrane proteins. First, following the treatment, the hydrophobic proteins (a small percentage of the original membrane content) are recovered in an organic solution containing 100% of the original lipids and pigments. Elimination (evaporation under nitrogen or argon) of the organic solvent allows a pellet containing all these components to be recovered. As a consequence, it is almost impossible to resolubilize these proteins in classical sample buffers and to analyse them on a classical SDS-PAGE. One option relies on the elimination of lipids and pigments using washes of the proteins/lipids/pigments pellet with cold acetone or isopropanol. This was demonstrated to be poorly efficient and lowers the yield of membrane proteins purified due to losing many proteins at the washing stage (Seigneurin-Berny et al., 1999). An alternative relies on the direct precipitation of hydrophobic proteins in the organic phase using four volumes of cold acetone (Ferro et al., 2002, 2003). This removes lipids and pigments from the protein sample and strongly limits the problems with resolubilization and migration of the hydrophobic proteins.

The proportion of poorly hydrophobic proteins of the purified membrane fraction can also be an important limitation. The proteins that are not soluble in the organic solvents are expected to precipitate. As a consequence, if the original membrane fraction contains too high a proportion of poorly hydrophobic membrane proteins and many hydrophilic contaminants, this induces the precipitation of all the proteins present in the original sample. The use of salt- or alkaline-treated membranes lowers the proportion of poorly hydrophobic proteins in the sample and thus limits the loss of hydrophobic proteins induced by precipitation of hydrophilic proteins.

The last major limitation concerns the yield of proteins extracted from the purified membrane fraction. This treatment is selective for highly hydrophobic proteins (Seigneurin-Berny et al., 1999), so most of the original content of the membrane vesicles, including some true integral membrane proteins, will be lost after this type of extraction.

Salt and alkaline treatments

Organic solvent treatment of plant membranes provided a new insight on the hydrophobic protein content of the membrane system under study. However, in the case of the chloroplast envelope membranes, this fraction only represents 5% of the total membrane proteins; therefore a more extensive survey is needed for a better view of the membrane proteome. For instance, alkaline and saline treatments of purified membrane fractions result in the extraction of different sets of proteins: Na2CO3 or NaCl extracts proteins that are rather weakly associated with the membrane whereas NaOH removes proteins that are more tightly associated (Fig. 1B).

Salt (NaCl, KBr, KNO3) treatments abolish electrostatic interactions of peripheral membrane proteins with the integral membrane proteins or the polar head of lipids. As a consequence, many peripheral proteins are eliminated after these types of treatments when most of the integral membrane proteins are still embedded in the vesicle bilayers. As stated above, sonication of the membrane vesicles in the buffer used for the salt treatment helps to remove peripheral proteins from the inner surface of membrane vesicles (50% of proteins to be removed). Non-sonicated chloroplast membrane vesicles still store some contaminating stroma proteins.

Alkaline treatments of membrane vesicles are expected to eliminate not only peripheral membrane proteins but also some lipid-anchored proteins and proteins that interact through hydrophobic interactions with integral membrane proteins or the polar head of lipids. As a consequence, many proteins are lost after these types of treatments and essentially true integral membrane proteins remain attached
to the vesicle bilayers. Sonication of the membrane vesicles in the buffer used for the alkaline treatment is essential to eliminate the proteins from both surfaces of membrane vesicles.

Such salt and/or alkaline treatments were successfully used for proteome analysis of plastid envelope membranes (Ferro et al., 2003; M Ferro, unpublished results), mitochondrial membranes (Millar and Heazlewood, 2003; Brugière et al., 2004), plasma membrane (Santoni et al., 1999; Marmagne et al., 2004), and thylakoid membranes (Friso et al., 2004).

Establishing protein repertoires of plant membranes

Using a wide range of methods is the only way of obtaining an exhaustive repertoire of a complex protein mixture. In most cases, membrane proteins separated by SDS-PAGE, are in-gel digested by trypsin and tryptic fragments are analysed by LC-MS/MS (Ferro et al., 2003) (Fig. 2). Combined with the use of different extraction procedures and analytical techniques, this allows the identification of proteins with different dynamic ranges and hydrophobicity. It is therefore important to use different fractionation methods to get access to the more minor proteins. For instance, Ferro et al. (2003) identified by proteomics the Pho2;1 protein in the SDS–PAGE band which contains the phosphate/triose-phosphate transporter. Whereas this last protein represents about 20% of the chloroplast envelope protein content, the Pho2;1 protein is only present at trace level and was only identified in the chloroform/methanol extract because it was extracted by the organic solvent and therefore concentrated in the organic phase.

Combining extraction procedures provides a complementary view of membrane proteomes

Using the different procedures described above, i.e. solubilization in chloroform/methanol, and alkaline and saline treatments, Ferro et al. (2003) identified more than 100 proteins in envelope membranes from Arabidopsis chloroplasts: about two-thirds (69/112) of the proteins identified were found from only one extraction method and not from either of the others.

As expected, organic solvent extraction gave the highest percentage of highly hydrophobic proteins retrieved. This extraction method allowed the identification of putative transporters, which were not detected using either NaOH or NaCl treatments and which are likely to be low abundance transporters. Although less stringent with respect to hydrophobicity, alkaline and saline treatments allowed the identification of a few highly hydrophobic proteins together with more hydrophilic ones. As expected, the most stringent extraction method led to the identification of the smallest number of proteins. Chloroform/methanol is the most stringent method and alkaline treatment is more stringent than saline treatment with regard to the recovery of hydrophobic proteins. Chloroform/methanol extraction, alkaline and saline treatments of Arabidopsis envelope membranes allowed the identification of 37, 51, and 74 proteins, respectively (Ferro et al., 2003). In addition, chloroform/methanol solubilized the most hydrophobic envelope proteins: almost all Arabidopsis envelope proteins identified in the chloroform/methanol extract have a res/TM ratio below 200 (31 among 37 identified proteins). As expected, more ‘soluble’ proteins (Res/TM above 600) were identified in NaCl-washed envelope membranes, even when compared with NaOH-washed membranes. These results provide further evidence that chloroform/methanol extraction is indeed a very powerful method to extract (and therefore concentrate) the most hydrophobic proteins of a membrane fraction for proteomic analyses.

As with plastid envelope membranes, the use of complementary extraction methods (i.e. chloroform/methanol extraction, alkaline and saline treatments) provided a rather broad view of proteomes from mitochondrial membranes (Brugiere et al., 2004) and plasma membranes (Marmagne et al., 2004). For the chloroplast envelope and mitochondrial membranes sets of data, less than 10% of proteins were identified from the three extraction procedures, while more than 60% of proteins were identified from only one type of extraction (Ephritikhine et al., 2004). Similarly, for the plasma membrane samples, a weak overlap was observed between these three extraction procedures, even though protein identification from the NaCl fraction was performed in a more exhaustive way (Ephritikhine et al., 2004). A difference in protein dynamic range and in
stringency, with respect to protein hydrophobicity, might explain the complementarities of the extraction methods, regardless of the subsequent analytical techniques used for protein identification. For instance, chloroform/methanol extraction allowed some minor and hydrophobic proteins to be concentrated and further identified, whereas these proteins were not identified from salt treatment experiments.

The weakness of overlap between extraction procedures must be tempered with respect to the subsequent identification process. For the above examples of envelope, plasma and mitochondria membranes, each fraction underwent one LC-MS/MS run only. If several runs had been performed for the same sample, more identification would have been obtained for each extraction procedure; which would certainly have increased the overlap. When analysing the same complex sample several times by LC-MS/MS, with the same analytical conditions, the identification list can be quite different. The most abundant proteins are always identified, but more minor proteins, for example, those identified by one or two peptides, can be identified from one run and not in another one. As for extraction procedures, MS analysis, as well as protein identification must be done several times and/or with different parameters to get complementary results and to increase the repertoire of identified proteins.

Physico-chemical properties of plant membrane proteins

As previously discussed by Ephritikhine et al. (2004), organic solvent extraction appears to select for low $M_r$ and high hydrophobicity with poor contamination by hydrophilic proteins in the case of the chloroplast envelope and the mitochondrial membranes. The situation is less clear-cut for the plasma membrane for which a higher percentage of hydrophilic proteins were identified in chloroform/methanol extracts. Plasma membranes behave somewhat differently from chloroplast envelope or mitochondrial membranes: whatever the extraction procedure used, the distribution of the physico-chemical properties displayed by plasma membrane proteins is spread over wider res/TM and $M_r$ ranges (Ephritikhine et al., 2004). In general, compared with chloroform/methanol extraction, NaCl treatment appears to be less efficient in identifying highly hydrophobic proteins: most of the proteins identified from saline treatment are predicted to contain no or only one transmembrane domain (Ephritikhine et al., 2004). NaOH treatment appears to be a good compromise to retrieve a wide range of proteins with different physico-chemical properties: this procedure clearly selects for more intrinsic proteins when compared with saline treatment since fewer hydrophilic contaminants are recovered (Ephritikhine et al., 2004).

Finally, in their comparative survey of plant membrane proteins extracted by chloroform/methanol, Ephritikhine et al. (2004) observed that the apparent selection of basic proteins by chloroform/methanol in the chloroplast envelope membranes (Ferro et al., 2002, 2003) is probably due to the nature of the proteins in this given membrane system. Whatever the extraction procedure used, most of the proteins identified from the plastid envelope, and especially proteins localized in the inner membrane, were shown to be basic (Ferro et al., 2003; Sun et al., 2004).

Validation of proteomic data: checking the subcellular location

Because the major interest of membrane proteomics is to bring functional clues that are tightly related to their subcellular localization, data validation is essential. This is true even though the membrane fractions analysed are highly purified. Of course, the plasma membrane localization of newly identified plasma membrane proteins was strengthened because of the presence of $H^+$-ATPase and PIPs in the plasma membrane fraction and also because of the lack of major proteins from other organelles, such as RuBisCO from chloroplast membranes or cytochrome c reductase from mitochondrial membranes (Marmagne et al., 2004). Two examples demonstrate the importance of the validation of the subcellular localization of membrane protein identified by proteomics in a given membrane system.

Firstly, during the course of proteomic analyses of the spinach chloroplast envelope, Ferro et al. (2002) identified a protein, namely the IEP60 $H^+$/Pi transporter, homologous to the Arabidopsis Phc2;1 Pi transporter. The Phc2;1 Pi transporter was previously suggested to be localized in the plasma membrane and to be involved in the uptake and intercellular movement of Pi in Arabidopsis shoots (Daram et al., 1999). In support of the proteomic analyses, experiments based on transient expression of Phc2;1::GFP fusions in Arabidopsis leaves and western blot analyses demonstrated unambiguously the localization of this protein in the inner membrane of the chloroplast envelope (Versaw and Harrisson, 2002; Ferro et al., 2003).

Secondly, the identification of proteins from the VDAC (Voltage Dependent Anion Channel) family in the plasma membrane proteome was unexpected (Marmagne et al., 2004), since it had also been described in mitochondrial membranes (Brugière et al., 2004). Although the dual localization of soluble proteins targeted to both mitochondria and plastids is supported by increasing data based on in silico sequence analyses combined with the molecular characterization of the targeting signals (Chew and Whelan, 2004), this is much more difficult for proteins for which clear targeting signatures have not yet been identified. In the case of AtVDAC1, the plasma membrane targeting was further confirmed (Marmagne et al., 2004) by transiently co-expressing in onion epidermal cells the translational fusions AtVDAC1::DsRed and Nramp3::GFP, a tonoplast
marker. These observations are also supported by studies of VDAC proteins in Lotus japonicus that seemed to localize not only to mitochondria but also to undefined vesicles in root nodules (Wandrey et al., 2004). Such a double localization of a VDAC channel is actually well accepted for animal models: the same protein VDAC can be targeted to both plasma membrane and mitochondria, depending on an alternative splicing in the 5′ UTR region (Buettner et al., 2000).

**Conclusions and outlook**

In summary, a whole set of methods are available to analyse membrane proteomes. A combination of different treatments of Arabidopsis cell membranes provide a broad view of some of the main Arabidopsis membrane proteomes. The strategy that has been described proved to be successful in identifying hundreds of membrane proteins, most of which are unknown and have no putative function. This was especially true for the most hydrophobic membrane proteins, which are putative transport proteins, for which very little information was available. To face the avalanche of proteomic data, the major goal in the very near future is to identify the functions of all the sets of proteins identified, the majority of which were previously unknown (Barbier-Brygoo et al., 2001). For instance, amongst the hundreds of proteins identified in the plasma membrane hydrophobic proteome (Marmagne et al., 2004), 95% had never been found in earlier proteomic studies.

Another key issue is that membranes are a dynamic system that reflects the diversity of tissues and their metabolic specificities. For instance, the protein equipment of plastid envelope membranes follows the structural and functional diversity of plastids (from proplastids to chloroplasts) in various cell types and tissues (leaves, roots, stems, flowers). Therefore, envelope proteomes are likely to be strikingly different in chloroplasts, proplastids, etioplasts, chromoplasts, etc. Such complexity illustrates that although far from having a complete picture of plant proteomes, proteomics is expected to continue providing new data in the near future. One of the key limitations is the ability to prepare highly purified membrane subfractions for the various tissues to be analysed.

*In silico* analyses of gene and protein structures using all the diversity of the bioinformatic tools may bring some clues for identifying the function of a membrane protein (see review by Ephritikhine et al., 2004). Although some tools are already able to analyse large protein repertoires, others have to be developed, especially those devoted to the systematic analysis of membrane protein structures (i.e., predictions of hydrophobic clusters, α-helices and β-barrel). In a phylogenetic profiling of the Arabidopsis thaliana proteome aimed at distinguishing plants from other organisms, Gutiérrez et al. (2004) observed that a significantly higher proportion of plant-specific proteins of unknown function were predicted to be membrane associated when compared with the known plant-specific proteins or with the entire proteome of Arabidopsis. This observation confirms (i) the interest of putative membrane-associated plant-specific proteins for future functional studies and (ii) the importance of bioinformatics for analysing plant membrane proteomes (see also Barbier-Brygoo et al., 2001; Ferro et al., 2002; Koo and Ohlrogen, 2002; Durand et al., 2003; Chew and Whelan, 2004; Friso et al., 2004; Sun et al., 2004; Vandenbrouck et al., 2005). For future progress, tighter links with bioinformatics should be set up: the functional characterization of the proteomes still lacks more accurate and reliable programs for functional annotation, especially in the case of membrane proteins. Progress should come from a combination of proteomic strategies applied to membrane subproteomes. Building up proteome repertoires combined with the development of methods for analysing membrane proteome dynamics need the development of new tools dedicated to membrane systems. The authors are convinced that progress in proteomics are closely related to those in transcriptomics and both are completely dependent on the capacity of the bioinformatics tools to handle and integrate the huge amounts of data generated by these approaches.

**References**


