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An evaluation of techniques for membrane proteomics in poorly sequenced plants: A case study on banana

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Summary

Banana is an important food crop for millions of people, but its cultivation is threatened by several types of biotic and abiotic stress. The reaction of a banana plant to these stresses depends on many factors of which one is the activity and presence of specific proteins. Proteins from poorly sequenced plants such as banana are usually studied by two-dimensional gel electrophoresis. However, the technique is not suited for the analysis of membrane proteins, due to the low abundance and poor solubility in aqueous media of this class of proteins. Since membrane proteins are often key-players in stress reactions, it was important to evaluate and optimize techniques that do allow the study of these proteins in banana.

Given the poor sequencing status of banana, protein based separation techniques were an obvious choice. Using these techniques, peptides that belong to the same protein remain connected during separation, which facilitates protein identification. Moreover, quantification at the protein level is generally more straight-forward compared to quantification at the peptide level. Currently, only gel-based methods for protein separation are described for membrane proteomics. Unfortunately, they are all limited in resolution, which makes a stringent enrichment step prior to protein separation an absolute requirement. Initially, a chloroform/methanol extraction was performed because this extraction method is known to preferentially extract highly hydrophobic proteins with short hydrophilic domains. Although membrane proteins could indeed be extracted from total cellular lysates, also a considerable amount of abundant soluble proteins were identified. Therefore, total membrane fractions were preferred as starting material for the evaluation of a second technique; i.e., blue native electrophoresis, a technique developed for the study of protein complexes. We evaluated whether this technique separates proteins that be-

long to a membrane protein complex from the bulk of proteins. In addition, we investigated whether the technique can be applied for the study of protein interactions in banana. Generally, blue native electrophoresis is combined with SDS-PAGE, but this approach is associated with protein losses during the transfer from the first to the second dimension. Therefore, we also evaluated the combination of blue native electrophoresis in the first dimension with gel-free peptide separation in the second dimension. The results of the latter approach were promising but further optimization is required. Moreover, the total membrane fraction still contained several abundant soluble contaminants which hindered the identification of less abundant membrane proteins. We concluded thus that membrane proteome studies require the use of an isolated organelle or membrane as starting material.

We focused subsequently on the plasma membrane, since this membrane forms the barrier between the cell and its environment. Proteins associated with this membrane are thus likely involved in the reaction to changes in the environment. The analysis of the plasma membrane proteins also revealed that the resolution of current gel-based techniques for membrane proteomics is limited which hinders the large-scale identification of membrane proteins. Therefore, we also evaluated gel-free, peptide-based, separation techniques, which implied that protein identification needed to be performed with the greatest care. We used a workflow based on *de novo* identification and visualization of protein inference.

Based on our findings, we could finally propose an efficient workflow for membrane proteomics in a poorly sequenced species. This workflow lays the foundation of the optimization of the quantitative aspect of membrane proteomics in banana.

Nederlandse samenvatting

Miljoenen mensen zijn afhankelijk van banaan als voornaamste voedselbron. De bananenteelt wordt echter bedreigd door verschillende omgevingsgebonden stressfactoren. De reactie van een plant op deze stressfactoren hangt af van verschillende elementen. Eén van deze elementen is de aanwezigheid en activiteit van bepaalde eiwitten. De studie van eiwitten in planten waarvan het genoom niet gekend is, gebeurt meestal met behulp van twee dimensionale gel elektroforese. Een groot nadeel van deze techniek is dat hij niet toelaat om membraaneiwitten te bestuderen. De belangrijkste redenen hiervoor zijn dat membraaneiwitten niet talrijk zijn en dat ze daarenboven niet goed oplosbaar zijn in een hydrofiele omgeving. Aangezien deze eiwitten een belangrijke rol spelen in verschillende fysiologische reacties, was het belangrijk om technieken te evalueren en te optimaliseren om het bestuderen van membraaneiwitten in banaan toch mogelijk te maken.

Aangezien slechts een heel beperkt deel van de genoomsequentie van banaan gekend is, waren technieken voor de scheiding van eiwitten een logische keuze. Peptiden die tot eenzelfde eiwit behoren blijven tijdens de scheiding met elkaar verbonden, hetgeen eiwitidentificatie vergemakkelijkt. Bovendien is het kwantificeren van eiwitten meer vanzelfsprekend in vergelijking met het kwantificeren van peptiden. Momenteel zijn enkel gel-gebaseerde technieken voor scheiding van membraaneiwitten beschreven. Helaas zijn deze technieken allen gelimiteerd in hun scheidingsvermogen. Hierdoor is een sterke verrijking in membraaneiwitten vóór de scheiding noodzakelijk. Eerst werd gedacht aan een extractie in een mengsel van chloroform en methanol aangezien deze methode specifiek sterk hydrofobe eiwitten met korte hydrofiele gebieden zou isoleren. Naast membraaneiwitten, werden er echter ook een aantal abundante niet-membraaneiwitten teruggevonden in het chloroform/methanol extract.

Daarom besloten we om bij de evaluatie van een tweede techniek, zijnde "blue native" elektroforese, te vertrekken van een totale membraanfractie. Deze techniek is ontwikkeld voor de studie van eiwitcomplexen. Tijdens de doctoraatsthesis werd hij geëvalueerd voor zijn capaciteit om membraaneiwitten te scheiden van de eiwitten die niet tot een complex behoren en als techniek om de interacties tussen eiwitten te bestuderen in banaan. In zijn meest algemene toepassing wordt "blue native" elektroforese gecombineerd met SDS-PAGE. Deze aanpak gaat echter gepaard met verlies van eiwitten bij de transfer van eiwitten van de eerste naar de tweede dimensie. Daarom werd ook getest of "blue native elektroforese" kan gecombineerd worden met de digestie van alle eiwitten die behoren tot één complex en een scheiding van de resulterende peptiden via chromatografie. Deze aanpak lijkt beloftevol maar vereist nog bijkomende optimalisatie. Zo werden nog steeds te veel abundante, niet-membraaneiwitten geïdentificeerd, hetgeen de identificatie van de minder abundante membraaneiwitten verhinderde.
Hieruit werd besloten dat een succesvolle studie op membraaneiwitten moet starten met het opzuiveren van een welbepaalde membraan. In dit onderzoek werd gekozen voor de plasma membraan omdat deze de scheiding vormt tussen een cel en zijn omgeving. Eiwitten die met deze membraan geasoccieerd zijn, kunnen dus een rol spelen bij het detecteren van stress in de omgeving en bij het tot stand brengen van een gepaste reactie. Aangezien de beschikbare gelgebaseerde technieken voor membraan proteomics eerder beperkt bleken in hun scheidingsvermogen, werden ook gel-vrije, peptide-gebaseerde scheidingstechnieken geëvalueerd. Dit houdt in dat de eiwit-identificatie met de grootste zorg moest gebeuren. Deze identificatie was gebaseerd op het aflezen van een eiwitsequentie van het massaspectrogram en op het visualiseren van de peptiden die aan eenzelfde eiwit zijn toegekend.
Gebaseerd op al de bekomen resultaten kon uiteindelijk een efficiënte werkwijze voor het bestuderen van membraaneiwitten in planten waarvan maar zeer weinig genoom-informatie beschikbaar is, geformuleerd worden. Deze werkwijze kan nu een basis vormen voor het optimaliseren van het kwantitatieve luik van membraan proteomics.

Symbols and abbreviations

Symb.-Abbr.	Description
16-BAC	benzyldimethyl-n-hexadecylammonium chloride
2-DE	classical two dimensional electrophoresis
ABC	ATP binding cassette
ACN	acetonitrile
Agi	*Arabidopsis* gene identifier
ALS	acid-labile surfactants
AmBic	ammonium bicarbonate
BN	blue native electrophoresis
BNE	blue native/SDS-PAGE
C/M	chloroform/methanol
CBB	Coomassie colloidal brilliant blue
CN	clear native electrophoresis
CTAB	cetyltrimethylammoniumbromide
DIGE	difference gel electrophoresis
DRM	detergent resistant membrane
dSDS	SDS/SDS-PAGE (doubled SDS-PAGE)
DTT	dithiothreitol
ER	endoplasmatic reticulum
ESI	electrospray ionization
EST	expressed sequence tag
FA	formic acid
FDR	false discovery rate
geLC	gel-based protein and liquid chromatography-based peptide separation
GRAVY	grand average hydropathy
GO	gene ontology
GPI	glycosyl-phosphatidylinositol
HPLC	high performance liquid chromatography
hrCN	high resolution clear native electrophoresis
ID	identifier
IMP	integral membrane protein
IPG	immobilized pH gradient

Symb./Abbr.	Description
ISO	inside-out
iTRAQ	isobaric tag for relative and absolute quantification
LC	liquid chromatography
LTCI	laboratory of tropical crop improvement
M_r	relative molecular mass
MALDI	matrix assisted laser desorption/ionization
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MudPit	multidimensional protein identification technology
NCBI	national center for biotechnology information
NR	non redundant peptides
OXPHOS	oxidative phosphorylation
pI	iso-electric point
PEG	polyethylene glycol
PIP	plasma membrane intrinsic protein
PM	plasma membrane
PPDB	plant proteome database
PTM	post-translational modification
PVPP	polyvinylpyrrolidone
Q	quadrupole
RLK	receptor like kinase
RP	reversed phase
RSO	right-side-out
SC	spectral counting
SCX	strong cation exchange
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide electrophoresis
SILAC	stable isotope coded labeling with amino acids in cell culture
S/N	signal-to-noise ratio
SPE	solid phase extraction
TCA	trichloroacetic acid
TFA	trifluoroacetic acid
TIP	tonoplast intrinsic protein
TMD	transmembrane domain
TMH	transmembrane helix
TOF	time-of-flight

Contents

Part I

Introduction

Rationale, aims and thesis outline

At the laboratory of Tropical Crop Improvement (LTCI) we aim at improving agriculture in the tropics and focus on banana farming. Bananas are perennial, evergreen, monocotyledonous, flowering herbs belonging to the genus *Musa* (family *Musaceae*, order *Zingiberales*). They are cultivated throughout the tropics and subtropics and constitute a major staple food crop in these regions. The majority of producers are small-scale farmers growing the crop either for home-consumption or for local markets. In their gardens, they grow several of the more than 1000 existing *Musa* varieties. Commercial plantations are focused on worldwide export and produce almost exclusively cultivars of the Cavendish subgroup (AAA) because of their long shelf life and the ability to control their ripening through ethylene. However, this monocropping is not without a risk since bananas are very susceptible to pests and diseases. The most effective and economical and environmentally friendly approach to control these threats is the use of resistant cultivars (Line and Chen, 1995). It is therefore important to safeguard the large *Musa* biodiversity and to explore its potential. At the laboratory of Tropical Crop Improvement a substantial part of this biodiversity is guarded in the largest *in vitro* collection of banana in the world under supervision of Bioversity International. To ensure safe, long term storage, efforts are now made to cryopreserve the whole collection. Cryopreservation overcomes disadvantages associated with *in vitro* storage such as the risk of somaclonal variation and human errors (e.g., contamination and mislabeling during labour intensive subculture events) (Vuylsteke et al., 1991). Almost all biochemical and physical processes will be arrested because of the ultra-low temperatures during storage, implying that cells or tissues can be

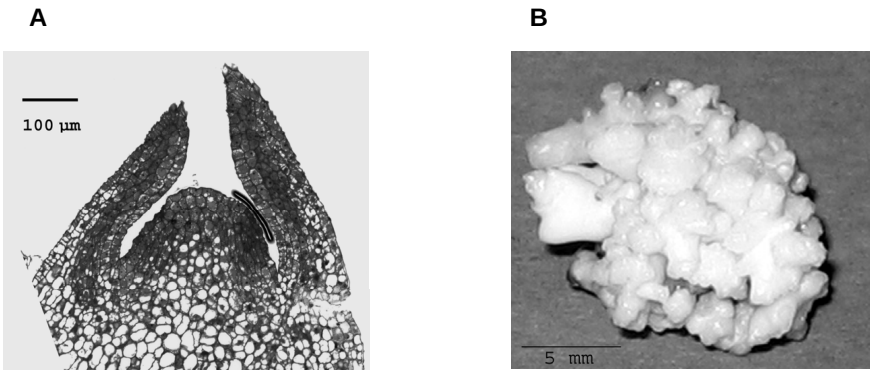

Figure 1: *A. Shoot tip with shoot meristem. B. A cauliflower-like banana meristem culture.*

maintained for unlimited periods. For the conservation of *Musaceae*, droplet vitrification of the apical meristems is preferred (Panis et al., 2005). Meristematic tissue consists of undifferentiated totipotent cells whose function is analogous to stem cells in animals. Shoot apical meristems (figure 1A) give rise to the above-ground parts of a plant. Another frequently applied cryopreservation protocol relies on preculture of proliferating meristems on media with high sucrose concentration (0.4 M). For this purpose, cauliflower-like shoot-tip meristem cultures were developed (Strosse et al., 2006) (see figure 1B). The post-thaw regeneration frequencies however are dependent on the genotype, ranging from 53% for ABB bananas to 3.9% for AAA bananas (Panis et al., 1996).

To improve the banana yield of local farmers in the tropics and subtropics, the underlying mechanisms of resistance toward several biotic and abiotic stresses are investigated by comparing varieties with a different degree of resistance (Carpentier et al., 2007). Main focuses lie on the resistance toward drought, fungi and nematodes. Despite the importance of banana as one of the most important food crops, it is still very poorly characterized. Currently, less than 1% of the genome is sequenced. The A genome is estimated at 638 Mb (11 chromosomes) and the B genome at 529 Mb (11 chromosomes) (Lysak et al., 1999). This situation will change in the future since Génoscope[1] and CIRAD[2]

[1] `http://www.cns.fr`
[2] `http://www.cirad.fr`

announced the sequencing of a homozygote AA banana cultivar (*Musa acuminata*, accession Pahang HD, 600 Mb, 11 chromosomes) in September 2009. The end of the operation is foreseen in 2011. In a comparison of a proteomics and transcriptomics approach it became clear that a sequenced genome is a prerequisite for high-throughput mRNA based approaches (Carpentier et al., 2008a). Therefore our group focuses on the proteomics part of the puzzle.

In **chapter 1**, several techniques that have been developed in the last decades to facilitate proteomics experiments are briefly discussed. In general, a proteomics experiment proceeds through the stages of protein extraction, separation and identification. For separation, two main approaches have been developed. In a first approach, proteins are separated before they are digested into peptides. This ensures peptides belonging to the same protein remain connected during separation which facilitates protein identification. In an alternative approach proteins are digested before separation. As such, after separation, it is not clear which peptides belong to the same protein. Therefore, the latter approach is mainly applied in proteomics studies of sequenced species such as *Arabidopsis thaliana* where one peptide can lead to protein identification. For proteomics studies of poorly characterized species such as banana, protein identification depends on the degree of similarity of banana proteins with proteins from sequenced plants. In this case, gel-based protein separation through classical two-dimensional electrophoresis (2-DE) is recommended. Using this 2-DE technique we could already demonstrate that several proteins are involved in the response on osmotic stresses. However, the main drawback of 2-DE is that the analysis of membrane proteins with more than two transmembrane domains is troublesome. Since membrane proteins are key-players in many physiological reactions, an important next step was thus the optimization of techniques for membrane proteomics in banana which is the goal of this PhD dissertation.

Membrane proteomics is challenged by the low abundance and poor solubility in aqueous media of membrane proteins compared to cytosolic proteins. In **chapter 2**, possible solutions for these problems are briefly discussed. From an overview of publications on plant membrane proteomics, it becomes clear that most studies have been performed on sequenced plants and include peptide based separations. Since banana is poorly sequenced, we had to explore the less frequently applied techniques that use gel-based protein separation. The

drawback of these techniques is that they are limited in resolution. Therefore, a stringent enrichment technique is required.

In **chapter 3** we use a mixture of chloroform and methanol (C/M) to specifically extract highly hydrophobic proteins with short hydrophilic domains from banana leaves and meristems. By performing a similar extraction on leaves of *Arabidopsis* and banana we demonstrate some of the difficulties that are associated with proteomics in poorly sequenced plants. It was the first time that this technique was applied on total cellular lysates. However, we had to conclude that the extraction on whole cellular lysates is less efficient with regard to the number of membrane proteins extracted, compared to an extraction on purified membranes.

In **chapter 4** we estimate the value of blue native electrophoresis (BN) as a method to selectively enrich samples in proteins that belong to a membrane protein complex and as a method to study protein-protein interactions in banana. For the first time, this technique is applied to study protein complexes in meristems. The use of blue native electrophoresis in its most popular application, i.e., in combination with SDS-PAGE, is associated with protein losses during the transfer from the first to the second dimension. Therefore, we evaluate the combination of protein complex separation using blue native electrophoresis and liquid chromatography based peptide-separation. As such, the final digested protein pool is highly reduced in complexity, and we could successfully identify banana proteins. This approach looks thus promising for future experiments. However, it was also clear that total membrane fractions still contain too many abundant contaminants to allow identification of less abundant membrane proteins.

Therefore, we focused on the plasma membrane (PM) proteome in **chapter 5**. This membrane is of particular interest because of its position at the interface between the cell and its environment. Proteins associated with this membrane are thus most likely involved in sensing environmental stresses and generating the appropriate response. Since the peptide-based approach looked promising when combining it with BN, we decided to evaluate whether it could be applied without the combination with a gel-based separation. Since gel-free peptide-based separations were not yet applied in membrane proteomics studies on

poorly sequenced species, we focus in this chapter on the workflow followed for protein identification. This workflow is based on *de novo* sequencing of unassigned mass spectra and the visualization of peptide-protein interactions to remove protein redundancy and false positive identifications.

Based on the acquired insights, we suggest a workflow for membrane proteomics in poorly sequenced plants in the **last chapter**. This workflow could encourage other researchers in the field not to stick to the study of soluble proteins but also to explore the membrane proteome. Moreover, it will be instrumental in the proteomics research on tolerance mechanisms to environmental stresses in banana plants. However, a major obstacle remains the performance of reliable quantitative studies of membrane proteins in poorly sequenced plants.

Chapter 1

The basic principles of proteomics

The main aim of this PhD dissertation is the optimization of techniques for membrane proteomics in poorly sequenced species to include them in the research on the mechanisms that determine the reaction of a banana plant to environmental stresses. To have a better understanding of the research domain proteomics, in this chapter a short overview of the techniques that have been developed for proteomics studies is provided. In the last part challenges for proteomics in a poorly sequenced plant such as banana are highlighted.

1.1 The proteomics building block

Proteomics is defined as the study of the proteome, the full complement of proteins expressed by a genome at a specific point in time (Wasinger et al., 1995). It includes not only the identification and quantification of proteins, but also the determination of their localization, modifications, interactions, activities and functions (De Hoog and Mann, 2004). Compared to genomics, proteomics has to deal with a higher degree of complexity since the proteome does not only change in response to physiological, developmental and environmental conditions, there also exists a considerable difference in concentration between the most and least abundant protein (i.e., the dynamic range). In addition, proteins can be post translationally modified with a change in function

as a consequence. Moreover, no amplification technique such as the polymerase chain reaction, which is used to amplify the least abundant gene transcripts, exists for proteins.

It is impossible to study the whole proteome in one single experiment. Therefore, a proteomics experiment has to be designed very carefully according to the biological questions that have to be answered. On the other hand, proteomics experiments can add crucial information to genomic and transcriptomic information because proteins are the real effectors of physiological reactions and the level of mRNA does not always correlate with the level of the corresponding proteins (Gygi et al., 1999). Moreover, protein amino acid sequences are more conserved than the corresponding nucleic acid sequences which makes proteomics a more valuable tool in studies of poorly characterized species like banana (Carpentier et al., 2008b). However, protein identification remains a difficult task a.o., because of the presence of post-translational modifications.

The ideal proteomics approach consists of a combination of high sensitivity, high throughput, analytical robustness, the ability to differentiate between differentially expressed proteins and the ability to analyze as many proteins as possible.

Briefly, a proteomics study proceeds through the phases of protein extraction, separation, analysis and mass spectrometric identification (Newton et al., 2004; Carpentier et al., 2008b).

1.2 Protein extraction

The extraction of proteins from their natural environment is the most critical step in any proteomics study because it is decisive for the final result. The ideal extraction method captures the greatest possible number of proteins from a biological sample and is compatible with downstream protein analysis (Saravanan and Rose, 2004; Maldonado et al., 2008). In plants, protein extraction generally starts with breaking the protecting cell wall and plasma membrane. Inevitably, also other intracellular components which interfere with subsequent proteomic analyses such as proteases, cell wall polyphenols, polysaccharides, starch, lipids, and various secondary metabolites are released. Obviously, these components have to be removed from the protein sample. Carpentier et al. (2005) compared several protocols that deal with this problem and found that phenol extraction

was the most powerful. Saravanan and Rose (2004) also proved that phenol extraction is preferable when working with recalcitrant tissue. To inactivate the (poly)phenols, dithiothreitol (DTT, which reduces the phenols by the formation of thioethers) or polyvinylpyrrolidone (PVPP, a strong H-acceptor which absorbs the phenols) is added to the extraction buffer. After extraction, proteins are kept in solution by addition of denaturating agentia (e.g. chaotropes) and/or (ionic) detergents to the solubilization buffer (Rabilloud, 1996; Rabilloud et al., 1997, 2009). The most widely applied chaotropes in proteomics are urea and thiourea. The best known and most effective ionic detergent is the anionic detergent sodium dodecyl sulfate (SDS).

When only a certain group of proteins is of interest (e.g., membrane proteins), a pre-fractionation technique needs to be applied. Various strategies have been developed over the years to fractionate proteins into sub-proteomes based on biochemical, biophysical, and cellular properties and are discussed by a.o., Ephritikhine et al. (2004), Rose et al. (2004), Righetti et al. (2005) and Bodzon-Kulakowskaa et al. (2007). The techniques used in membrane proteomics will be discussed in more detail in chapter 2.

After protein extraction, proteins have to be separated from each other in order to analyze them individually.

1.3 Separation

Two main workflows for separation can be distinguished. In the first approach proteins are separated, generally using gel electrophoresis, before digestion into peptides. In the second approach proteins are generally first digested into peptides which are subsequently separated using chromatographic techniques. However, sometimes intact proteins are separated through liquid chromotagraphy and subsequently fragmented in the mass spectrometer. This top-down approach is less frequently applied, mostly in studies on post-translational modifications (Doerr, 2008) and will not be discussed further.

1.3.1 Gel-based protein separation

The workhorse for gel-based proteomics is two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (2-DE), which was established by O'Farrell in 1975 (O'Farrell, 1975) (figure 1.1).

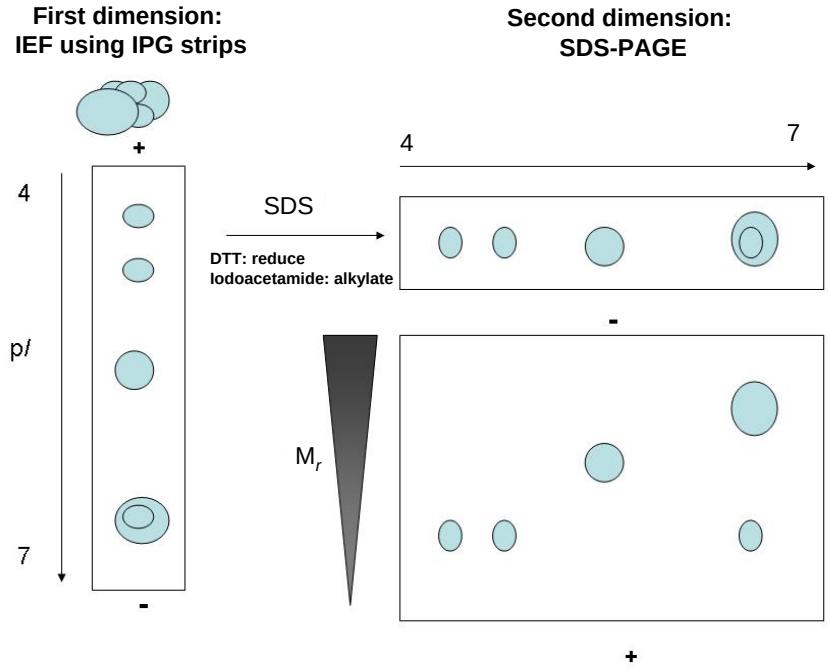

Figure 1.1: *Mechanism (simplified) of classical two-dimensional electrophoresis (2-DE). In the first dimension (left), proteins are separated according to their iso-electric point (pI). After addition of SDS, reducing and alkylating agentia, they are separated according to their molecular size (M_r) (right).*

In the first dimension, proteins are separated according to their intrinsic charge by means of an iso-electric focusing step. Proteins move in an electric field until they reach the point where their net charge is 0, i.e., their iso-electric point (pI). The development of immobilized pH gradient (IPG) strips greatly improved the reproducibility and ease of use of this step (Bjellqvist et al., 1982). High resolutions can be obtained by using IPG strips with narrow pH ranges (e.g., 1 pH unit) (Hoving et al., 2000, 2002). After the run, the strip is put on top of a polyacrylamide gel in which the second dimension separation occurs. Prior to this separation the proteins are "coated" by SDS molecules. The detergent aggregates at hydrophobic protein sites and induces protein denaturation. The resultant protein-detergent complexes consist of helical SDS-coated polypeptide regions separated by uncoated linkers, termed "necklace and bead" structures

(Shirahama et al., 1974). Maximum SDS-binding levels are generally estimated at 1.4 g SDS/g protein (Reynolds and Tanford, 1970). However, actual values vary between 1.5 and 2 g detergent/g protein (Tanford, 1980). This SDS coating masks the actual charge of the protein and renders all protein-detergent micelles with a negative charge. Therefore, they will be attracted to a positive charge from the electrode located at the gel end. Due to the molecular sieving effect of the polyacrylamide pores, this results in a migration and separation according to molecular size. The resolution of this step can be optimized by using acrylamide concentration gradients.

On average, 1000 to 3000 proteins can be quantified on one gel (20-24 cm), which is remarkably lower than the total number of proteins present in a sample. Wilkins and his colleagues estimate that to be detected proteins need to be present in at least 1000 copies/cell (Wilkins et al., 1998). Proteins with a lower abundance can be visualized by using a pre-fractionation technique, e.g., the removal of very abundant proteins such as Rubisco, and by loading higher sample amounts. Also highly hydrophobic or basic proteins are difficult to detect on 2-DE gels (Santoni et al., 2000; Wilkins et al., 1998). They require an alternative technique (protein- or peptide-based separation) which will be discussed in the next chapter. There are also limitations with respect to protein size. Resolution of proteins below 10-14 kDa is not sufficient because molecules smaller than 10 kDa co-migrate with the SDS front. This can be solved by using the Tricine SDS-PAGE system developed by Schägger and Von Jagow (1987). Also high molecular weight proteins are not well resolved on classical 2-DE gels. A solution is the use of agarose instead of polyacrylamide gels (Oh-Ishi et al., 2000). Because of the denaturing nature of the 2-DE technique, no information about protein-protein interactions is obtained. Therefore a non-denaturating gel-based technique, blue-native electrophoresis (BN) has been developed (Schägger et al., 1994), which will be discussed in section 2.4 on page 47.

Despite these limitations, classical 2-DE has many advantages. It is a very robust, highly reproducible technique under various experimental conditions, relatively easy to perform and no expensive equipment is required. A high resolution can be achieved, although some spots will still contain more than one protein. The biggest advantage of this high resolution is the ability to detect protein isoforms, including post-translational modifications (Rabilloud, 2002). However, it remains a labour-intensive technique that is hard to automate.

When a more high-throughput and automated workflow is required, a gel-free, peptide-based approach is the technology of choice.

1.3.2 Gel-free peptide separation

In a gel-free approach proteins are first converted into peptides using an enzymatic digestion step. The resulting peptides are generally separated by means of high performance liquid chromatography (HPLC). Several chromatographic columns can be coupled to each other to enhance resolution. The most widely used combination is a strong cation exchange column (SCX) coupled to a reversed phase (RP) column. On the first column, peptides are separated according to their charge, while their hydrophobicity is decisive for elution from the second column. Yates and his colleagues introduced this multidimensional protein identification technology (MudPit) in the beginning of this century (Washburn et al., 2001) (figure 1.2).

The columns can easily be coupled to a mass spectrometer, creating a high-throughput workflow. Depending on the type of mass spectrometer, a very high sensitivity can be reached. An alternative is the RP-RP LC system introduced by Gilar in 2005, where peptides are separated using two different pH's for elution from the first and second RP column (Gilar et al., 2005). According to the authors, the advantages of this approach are (i) after optimization, the number of peptides divided in several consecutive fractions is very limited (ii) the peptide losses in the first dimension are much smaller compared to SCX separation and (iii) the mobile phases are salt free and thus MS compatible. Moreover, the theoretical peak capacity is higher.

The main disadvantage of a peptide-based separation is that proteins are digested before separation which significantly enhances complexity of the sample and makes it hard to predict which peptides belong to the same protein. As a consequence, one peptide has to be decisive on its own for protein identification which makes its applicability on poorly sequenced organisms more troublesome. When peptides are shared between multiple proteins, it is impossible to obtain a protein identification based on these peptides (i.e., the protein inference problem). Therefore, protein separation is preferred for the study of protein isoforms. Another disadvantage is that the high solubilizing power of SDS can not be used unless the detergent is removed before separation by e.g., the filter aided sample preparation method of Wisniewski (Wisniewski et al.,

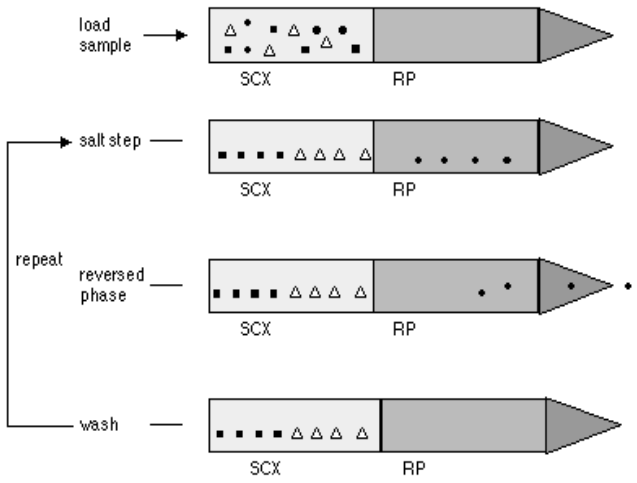

Figure 1.2: *The MudPit technology. Proteins are loaded on a strong cation exchange (SCX) column. They are unloaded by increasing salt concentrations (e.g., ammonium acetate). After each salt wash, they will bind on a reversed phase (RP) column that is coupled to the SCX column. Proteins are eluted from this column by increasing concentrations of a volatile organic solvent (e.g., acetonitrile). By combining two different chromatographic separations, a higher resolution can be achieved.*

2009) (see also chapter 5) since detergents are not compatible with subsequent tryptic digestion or mass spectrometric identification (see also section 2.3.1.1).

1.3.3 A combination of a protein and peptide separation

To take advantage of the properties of both gel-based protein and gel-free peptide separation, the two techniques can be used in a geLC approach. Proteins are first separated according to their molecular size through SDS-PAGE. Since only a limited number of proteins can be resolved in a single dimension (Williams et al., 2006), further separation is needed for complex samples. Therefore, in the geLC approach the gel lane containing the proteins is excised and divided into slices. After removal of the detergent and in-gel digestion of the proteins, the resulting peptides are separated using a RP column which can be on-line coupled to a mass spectrometer. Main advantages are that the

strong solubilizing power of SDS is used for protein solubilization and that a semi-automatic workflow is achieved. The final peptide mixture present in one LC run is significantly reduced in complexity which makes the chance of identifying low abundant proteins higher. On the other hand, the method is not very suitable for quantitative studies.

1.4 Protein quantification

One aim of a proteomics experiment can be to identify as many proteins as possible from a certain species, tissue or subcellular structure to generate a proteome map. However, the most interesting aspect of proteomics is the ability to compare the composition of different proteomes, thereby identifying proteins of which the concentration and/or the degree and nature of modifications differ. This requires that proteins are quantified, either relatively or absolutely. Relative quantification defines the amount of a protein relative to another measure of the same protein in another state. Absolute quantification determines the absolute amount of the protein in the sample. Since the aim of this dissertation is the optimization of techniques for membrane proteomics in poorly sequenced plants, no quantitative studies will be described. The current available techniques for quantification will therefore only briefly be described. More information on quantitative aspects can be found in excellent reviews of Panchaud et al. (2008); Riederer (2008) and Vaudel et al. (2010).

1.4.1 Quantification at the protein level

In the gel-based approach, protein spots are initially visualized. Miller and her colleagues nicely reviewed the different types of stain available for protein staining (Miller et al., 2006). The two most popular staining techniques are Coomassie colloidal brilliant blue (CBB) (Neuhoff et al., 1988) and silver based protocols (Switzer et al., 1979) because they are easy to use and have relatively low costs. The main advantage of CBB staining is its high compatibility with subsequent mass spectrometric protein identification. The main drawback is the high protein amounts it requires (i.e., 10-100 ng depending on the protocol) (Patton, 2002; Vercauteren et al., 2007). Silver staining protocols are more sensitive (1 ng) but have a low compatibility with current mass spectrometry (MS) protocols and are more time demanding (Rabilloud, 1990, 1992; Swain

and Ross, 1995). Therefore, silver is often preferred for analytical analyses, while CBB is used for preparative gels. Alternatives for these two conventional stains are fluorescent dyes such as the SYPRO dyes developed by Molecular Probes (Eugene, OR, USA). They are as sensitive as the silver staining methods, but superior to them in terms of ease of use, linear dynamic range (three orders of magnitude) and MS compatibility. Their main disadvantages are their higher costs and the need of more specialized detection apparatus.

The above described dyes are all applied after electrophoresis, thereby limiting the proteome that can be detected in one gel to one. Proteins can also be labeled prior to electrophoresis. In difference gel electrophoresis (DIGE) -based proteomics, the experimental and control samples are derived with different fluorophores and are run in the same gel, thereby minimizing technical variation (Unlu et al., 1997). Three CyDyes are commonly used, namely Cy-2, Cy-3 and Cy-5, which allows labeling of two samples plus an internal standard (Alban et al., 2003). The drawback of the DIGE technology is that it is very cost demanding and that it requires specialized equipment and evaluation software.

Certain post-translational modifications can be detected by specific stains of which the ones to detect glycosylated (e.g., Pro-Q Emerald® from Molecular Probes) or phosphorylated proteins (e.g., Pro-Q Diamond® from Molecular Probes) are the most widely applied. These stains can be applied sequentially so the different post-translational modifications can be visualized in one gel which eliminates gel-to-gel running variations. After staining, quantitative analysis of the stained protein spots is achieved through dedicated image analysis software (Rose et al., 2004).

1.4.2 Quantification at the peptide level

Quantification at the peptide level either occurs by adding a label or label-free. Label-based quantification relies on the incorporation of isotopic mass tags. These tags introduce predictable peptide mass differences that are easy recognizable in MS (MS^1) and MS/MS (MS^2) spectra (see section 1.5). The most common methods are nicely reviewed by Beynon and Pratt (2005), Vaudel et al. (2010) and include metabolic and post-metabolic labeling techniques. Metabolic labeling generally occurs through stable isotope coded labeling with amino acids in cell culture (SILAC) (Ong and Mann, 2006; Mann, 2006) or the variant stable isotope labeling in planta (SILIP) (Hebeler et al., 2008; Schaff

et al., 2008). Isotopes are incorporated post-metabolically by a chemical reaction or by enzymes. Chemical labeling methods include the isotope coded affinity tag (ICAT) technique (Gygi et al., 1999) and the isotope-coded protein labels (ICPL) method where the label is detected in the MS^1 spectra (Schmidt et al., 2005; Kellermann, 2008). In the isobaric tag for relative and absolute quantification (iTRAQ) technology (Ross et al., 2004), only after fragmentation the different labels become visual in the MS^2 spectra. Another approach is to add no label and to use the mass spectrometer output for quantification. Also in this approach, quantification occurs at the MS^1 or MS^2 level. Quantification at the MS^1 level is based on the measurement of the peak volume or peak intensity of a certain peptide ion and compare it between multiple runs. The peak intensity is proportional to the concentration of the peptide in the sample. When quantification is performed at the MS/MS level, the spectral counting method is the most popular. In this approach the frequency peptides are selected for further fragmentation is used as measure for relative protein abundance. An increase in protein abundance typically results in an increase in the number of its proteolytic peptides, which enhances the chance on further fragmentation (Liu et al., 2004).
When comparing two samples, only careful statistical analysis can define a protein as statistically different. Advice to perform reliable, statistics based proteomics given by Carpentier et al. (2008c).
For identification of proteins of interest, mass spectrometry is preferred (Aebersold and Mann, 2003).

1.5 Mass spectrometry

A mass spectrometer measures the mass-to-charge ratio (m/z) of gas-phase ions (Griffiths and Wang, 2009). It consists of an ion source for gas-phase ion generation, a mass analyzer that measures the m/z of the formed ions and a detector that registers the number of ions at each m/z value. The most popular ionization techniques are matrix assisted laser desorption/ionization (MALDI) (Karas and Hillenkamp, 1988) and electrospray ionization (ESI) (Fenn et al., 1989) (figure 1.3).
In MALDI (figure 1.3, upper part), peptides are co-crystallized with matrix molecules on a MALDI plate. When a pulse of laser hits this mixture of sample and matrix, energy of the laser is absorbed by the matrix molecules and

transferred to the peptides which subsequently ionize. The exact mechanism of how this ionization works is still not fully understood. The MALDI technique is mostly combined with the gel-based approach because of the low complexity of the sample to be analyzed (one or only a few proteins). However, also the fractions collected from LC based peptide separation are sometimes analyzed by MALDI-MS. During ESI (figure 1.3, lower part), the sample is dissolved in a polar, volatile solvent and pumped through a narrow capillary. A high voltage is applied to the tip of the capillary. Because of the charge, the droplets become unstable and fall apart into a cloud of tiny, highly charged droplets at the tip of the needle. This process is aided by a nebulizing gas (often nitrogen) flowing around the outside of the capillary. Gradually, the charged droplets diminish in size until all solvent is evaporated. These volatile ions pass through a sampling cone into a vacuum region. Subsequently, they enter the mass analyzer. Since HPLC columns can be on-line coupled to the spraying needle, ESI is preferred in the gel-free, peptide-based approach. This so-called shotgun method (Wolters et al., 2001) is currently the most popular proteomics workflow because of its high degree of automation.

After the ionization process, the formed gas-phase ions are separated in mass analyzers according to their mass-to-charge ratios. Five basic types of mass analyzers are used in proteomics (Schaeffer-Reiss, 2008; Griffiths and Wang, 2009): quadrupole mass-filters (Q); ion traps (IT); Fourier transform ion cyclotron resonance (FT-ICR), time-of-flight (TOF) and the recently developed orbitrap. They can work as stand-alone mass analyzer, but they are often coupled in order to combine advantages of different analyzers in one mass spectrometer. Generally, two mass analyzers which are separated by a collision cell are combined to form a tandem mass spectrometer (MS/MS). In the first mass analyzer the m/z of so-called precursor ions is determined. Then, ions with a specific m/z are selected and further fragmented in the collision cell through collision with an inert gas or by applying vacuum. The resulting fragment ions are analyzed in the second mass analyzer. The final element of the mass spectrometer is the detector which registers the number of ions at each m/z. Several types of detectors exist, dependent on the type of analyzer. The more common ones are the photomultiplier, the electron multiplier and the micro-channel plate (Schaeffer-Reiss, 2008). Because the number of ions leaving the mass analyzer at a particular moment is generally quite small, significant amplification is often necessary to obtain a usable signal.

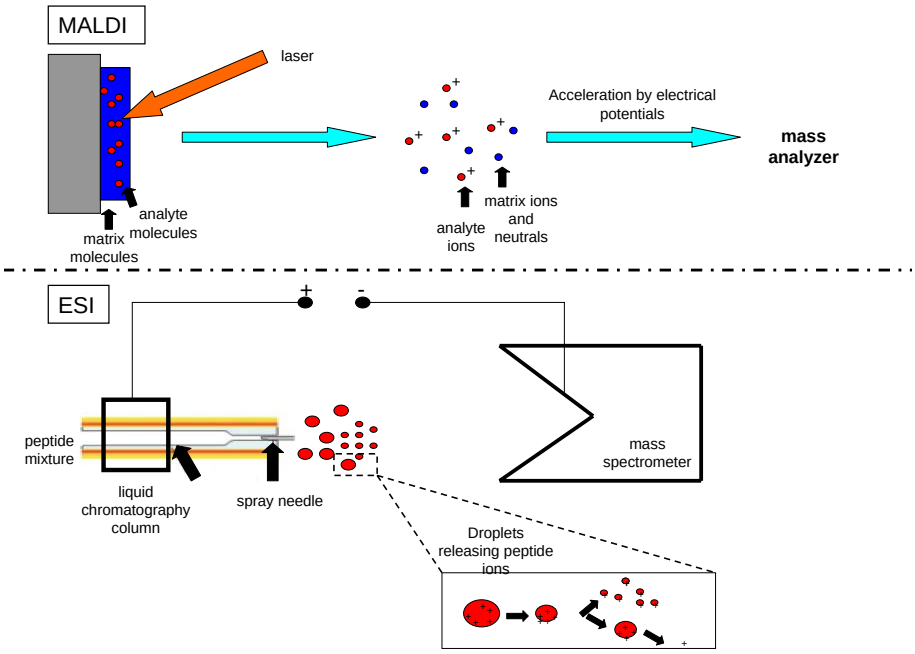

Figure 1.3: *The principles of the two soft ionization techniques matrix assisted laser desorption ionization (MALDI) and electrospray ionization (ESI). Adapted from Steen and Mann (2004).*

The final component of a mass spectrometer is the data system where the signal is converted into a mass chromatograms where the m/z value is shown on the x-axis and peak intensity on the y-axis. These chromatograms form the basis for peptide and protein identification.

1.6 Protein identification

Three main approaches are distinguished to assign a peptide sequence to a mass spectrum. These peptide identifications finally lead to the identification of the proteins the peptides are derived from.

1.6.1 Database searching

In the first approach, experimentally acquired spectra are correlated with theoretical spectra that are *in silico* generated from protein sequences present in a database (Griffiths and Wang, 2009). Several algorithms are developed to facilitate and automate this database search. The two best known programs are Mascot[1] and Sequest[2]. In the coming chapters Mascot is preferred for database searching because it is an open access program and because Elias and his colleagues demonstrated it has a better performance in combination with TOF instruments (Elias et al., 2005). Any FASTA format database, even nucleic acid databases being translated in six reading frames, can be searched. The entries in these sequence databases are theoretically cleaved with the same enzyme as used during the experiment by applying the appropriate cleavage rules. Generally trypsin is used which cleaves peptide chains at the carboxyl side of the basic amino acids lysine (K) or arginine (R), except when they are followed by a proline (P) (Steen and Mann, 2004). The cleavage site is not only easily recognizable, it also produces peptides of which the charge is easily predictable. Using MALDI, peptide charge will be generally +1 while using ESI it will be +2 (Wattenberg et al., 2002). The search can be performed on the MS or MS/MS level, respectively called peptide mass fingerprinting (PMF) or peptide fragment fingerprinting (PFF) (Aebersold and Mann, 2003).

In the PMF approach, peptide masses are compared with the theoretical masses of all the peptides, obtained after *in silico* digestion of a selected protein database. In practice, one measured mass matches to several combinations of amino acid sequences, which makes the method not suitable for the identification of complex protein mixtures. Therefore, PMF is generally applied for the identification of proteins from spots of 2-DE gels from completely sequenced species. In this case, all measured masses can exactly match to all theoretical masses of an *in silico* digest of a specific protein. Since the chance that a complete protein sequence is conserved between species is small, the method alone can not be used for studies of poorly sequenced species. They require an additional peptide fragment ion search. In this approach, all peptides of which the theoretical mass matches to the experimentally measured mass are further fragmented *in silico*. This fragmentation (see figure 1.4) depends on the amino

[1] freely available at `http://www.matrixscience.com/`
[2] commercially available from Thermo Finnigan

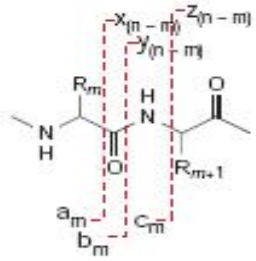

Figure 1.4: *The different types of fragmentation ions that can be formed, named according to the Roepstorff-Fohlmann-Biemann nomenclature. Ions containing the original amino terminus are called a_m, b_m, c_m in which m represents the number of R groups the ions contain; those containing the carboxy terminus are called $z_{(n-m)}$, $y_{(n-m)}$ and $x_{(n-m)}$ with (n-m) representing the number of R groups (n= total number of R groups in the peptide). Adapted from Steen and Mann (2004).*

acid sequence of the peptide and the used ionization method and mass spectrometer (Tabb et al., 2003, 2004). The preferential fragmentation behavior is explained by the "mobile" proton model (Dongré et al., 1996). According to this model, the proton responsible for fragmentation is able to move along the peptide backbone and to protonate any of the amide bonds, which then become susceptible to fragmentation. Because ESI is performed under more acid conditions compared to MALDI, the carboxy terminus of tryptic peptides will be protonated while in MALDI it will be deprotonated. This implies that when using ESI, y-ions and low mass b-ions are formed, while fragmentation after MALDI results in a series of y, b and a ions (Wattenberg et al., 2002).

After database searching, the applied searching algorithm will provide a significance score for the obtained protein and peptide matches. The determination of this score depends on the applied search engine. Sequest uses a spectral correlation function while Mascot reports the probability that the observed match has occurred by chance (i.e., the MOWSE score (Perkins et al., 1999)). There are no strict rules for setting a significance threshold, but generally it is the value corresponding to a chance of one in twenty ($p < 0.05$). Also the number of ion scores (peptide matches) that need to exceed the cut-off value and the number of matched peptides per protein is arbitrary.

For sequenced organisms, protein identifications based on the matching of two or more peptides with significant ion scores, are generally automatically reported. However, Gupta and Pevzner (2009) advises not to rely on this two peptide rule but on the false discovery rate (FDR) expectations (see subsection 1.6.4).
In an alternative approach, spectrum databases instead of sequence databases are searched (Frewen et al., 2006; Craig et al., 2006). These databases consist of previously generated mass spectra of the same or of a closely related species. An unknown spectrum can then be identified by comparing it to all the candidates in the spectral library to determine the match with the highest spectral similarity. Several research groups indicate that such spectrum databases lead to increased and more sensitive peptide identifications (Ahrne et al., 2009; Yen et al., 2009). However, peptides that were not previously entered into the database cannot be identified. Therefore, this method is currently used as a first rapid screening.

1.6.2 *De novo* sequencing

In the *de novo* approach, the spectra of the fragment ions are used to deduce the amino acid sequences by measuring the distances between two neighboring peaks. Interpretation can be performed manually, but there exist several programs that automatically deduce the amino acid sequence of a peptide such as Peaks (Ma et al., 2003), PepNovo (Frank and Pevzner, 2005) and Novo HMM (Fischer et al., 2005). The main advantage of *de novo* sequencing is that it allows the identification of peptides of which the exact peptide sequence is not present in the searched sequence database. It is therefore mainly used for protein analysis in species for which no or limited genome sequence information is available or for identifying modified peptides. Obviously, spectra of high quality (i.e., low background spectra) are required. To improve the quality of the spectra, background peaks can be removed. An overview of the current filtering strategies is provided by Salmi et al. (2009). Another development to facilitate reading of the obtained spectra is by producing only one type of ion series (e.g., Samyn et al. (2004), Sergeant et al. (2005)). An overview is given by Lu and Chen (2003) and Seidler et al. (2010). Reliable *de novo* sequences are searched against a protein database using an error tolerant search tool (i.e., a search that allows one or more mismatches) that allows identifying peptides on the

basis of close homology to peptides in the database (Grossmann et al., 2007; Waridel et al., 2007). Since this is a computationally time demanding task, most researchers first perform a database search and apply *de novo* sequencing tools to the remaining unassigned high quality spectra (see also materials and methods of chapter 5).

1.6.3 Hybrid approaches

Protein identification is sometimes performed by a combination of elements of the two above described methods. The analysis starts with the determination of a short sequence tag from a MS/MS spectrum, followed by an error tolerant database search. This method was first described by Mann and Wilm (1994) and is mainly applied to analyze post-translational or artifactual modified peptides (Tanner et al., 2005).

1.6.4 Evaluation of the identification

As mentioned above, one of the major problems associated with protein identification based on search engines is that they might return false positive results. To separate correct from incorrect matches, spectra can be manually verified and validated. However, this is subjective and depends on the level of expertise of the validating individual. Modern approaches have moved toward probabilistic approaches that provide a statistical measure of the confidence of the identification and that give an estimation of the error rate. They can be grouped into two main categories, but the best strategy is to use a combination of both methods that are described below. The final goal is to end-up with a list of identified proteins with a controlled FDR.

1.6.4.1 Target-decoy searches

A decoy-database is a database with the same size as the original search database but with the least possible peptide sequences in common. This is generally achieved by reversing the original protein sequences (Peng et al., 2003). The target-decoy strategy involves two steps. In a first step a search is performed against the target database augmented with the decoy database. Sometimes both searches are performed separately. This approach assumes

that false matches to the target database and matches to the decoy database follow the same distribution. In the second step the number of false positives is estimated. The false discovery rate is determined by $2D/N$ with D being the match to decoy sequences above the threshold determined by the software used, and N the number of all matched peptides above the cut-off value (Nesvizhskii et al., 2007). The advantage of this approach is that it is simple to implement in the currently available database search engines. Disadvantages are a doubling of the database search time and the question whether reversing or randomizing sequences provides an accurate estimation of the distribution of false peptide matches. A more detailed overview of this approach is given by Elias and Gygi (2007).

1.6.4.2 Empirical Bayes approaches

An alternative approach is based on a statistical model that validates the retrieved peptide and protein identifications. This model is integrated in online tools such as PeptideProphet for the validation of peptide identifications and ProteinProphet for the validation of protein identifications. Both tools are integrated in the trans-proteomic pipeline[3]. The programs combine different parameters to generate a new search score. The scores are determined for each search engine separately using training datasets with a known number of false positive identifications. The score is set as a cut-off value which varies according to the desired number of possible false positives allowed.

1.7 Challenges for poorly sequenced plants such as banana

With its poorly characterized genome, banana is an excellent example of a poorly sequenced plant. Proteomics on poorly sequenced organisms is not as straight-forward as proteome studies of fully characterized (model) species. To date only 8 plants are fully sequenced of which *Arabidopsis thaliana* (120 Mb, 2n=10) and *Oryza sativa* (450 Mb, 2n=24) are annotated to the most extent (NCBI Plant Genomes Central website). The specific technical challenges in

[3]freely downloadable at `http://sourceforge.net/projects/sashimi/files/`

proteomics studies on poorly sequenced species are reviewed by Carpentier et al. (2008b).

In the specific case of banana, initial problems arise during protein extraction. Since banana contains extremely high levels of oxidative enzymes (Gooding et al., 2001), phenolic compounds (simple phenols, flavonoids, condensed tannins, lignin) and high levels of latex and carbohydrates, a very stringent extraction protocol was developed. Phenol extraction first removes most of the interfering components. Subsequently, an excess of DTT is added to eliminate the (poly)-phenols through the formation of thio-ethers. Then, proteins are precipitated by the addition of ammoniumacetate and methanol. In this step, remaining lipids and pigments are removed (Carpentier et al., 2005). This protocol proved to be successful for several other plant species as well such as apple, pear, potato and *stevia*.

For protein separation, classical 2-DE combined with cross-species identification was the method of choice. Especially the high resolving power of this technique is of great importance for a poorly characterized species. In case the resolution is too low, multiple proteins are digested simultaneously resulting in a complex peptide pool in which peptides that are derived from the same protein can no longer be associated with each other (i.e., the identification problem). Consequently, a combination of masses of non-related peptides is submitted to a database search, thereby increasing the risk of generating false positive identifications. As already mentioned, this is especially true in peptide-based approaches since the total protein extract is digested prior to separation. Additionally, proteomics studies on poorly sequenced plants require the application of tandem mass spectrometry (MS/MS) to retrieve reliable identifications (Carpentier et al., 2008b). In the MS mode, peptides with a high signal-to-noise (S/N) ratio are selected for further fragmentation to obtain more informative data on the parent ion mass, enlarging the probability of obtaining significant hits. The S/N ratio of a peptide not only depends on the abundance of the protein from which it is derived from, but also on its ease to ionize and on its m/z. For this reason, peptides with the highest S/N ratio in a complex peptide mixture might be derived from different proteins. In case such intense peptides are less or not informative (i.e., not conserved in a sequenced species), identification will fail since more homologous peptides with a lower S/N ratio will not be selected for MS/MS.

However, when the peptide mixture consists of a limited amount of peptides which are all derived from a single protein, the informative peptides with a lower S/N ratio have a bigger chance of being selected for MS/MS, making protein identification possible (figure 1.5).

When only relying on a cross-species database search for protein identification, an identification rate of approximately 40% can be achieved (Carpentier et al., 2008b). Fortunately, also a banana specific library of approximately 32 000 entries exists. Searches against this database generally result in 15% additional identifications. To enhance the identification rate further, *de novo* sequencing can be applied (e.g., Samyn et al. (2007)). The workflow followed in a banana proteomics experiment is shown in figure 1.6.

In summary, this chapter gives an overview of the most common techniques applied in gel-based or gel-free proteomics. In their classical form, they display a few shortcomings, such as the lack of identification of basic and membrane proteins. Adaptations to the classical protocols made for membrane proteomics will be discussed in the next chapter.

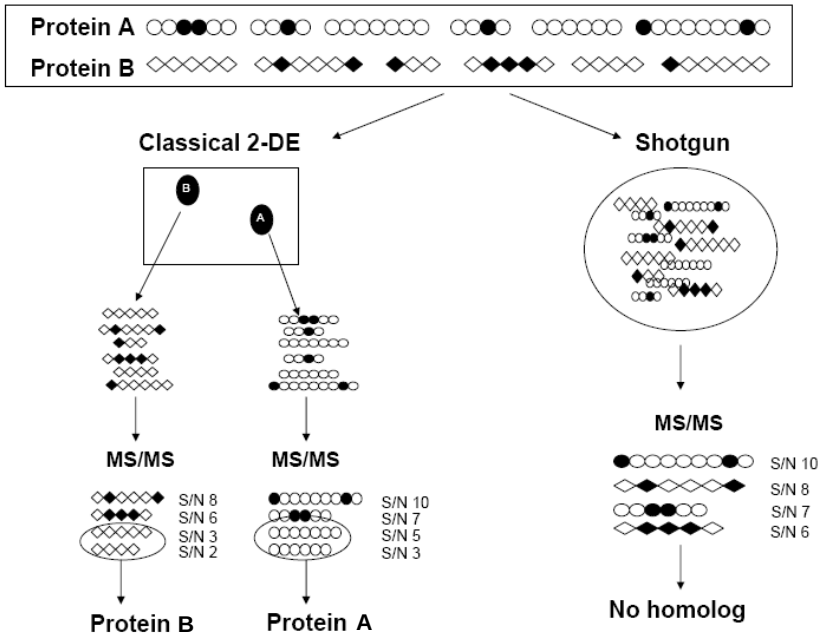

Figure 1.5: *A simplified overview of the identification/abundance problem in a shotgun approach on poorly sequenced plants. Two proteins (A and B) are digested into peptides (top). The symbols represent different amino-acids. Dark colored symbols indicate amino acid mutations that occurred in the poorly sequenced plant and not in the peptide sequences from sequenced homologous proteins. Protein identification in the gel-based and gel-free approach is demonstrated. During MS/MS the 4 most intense ions were selected for further fragmentation. This leaded to identification of both proteins in the gel-based approach, but none of the proteins could be identified using the gel-free approach because only modified peptides were selected for further fragmentation. Only when the peptide sequences are present in a species specific database, when amino acid substitutions are allowed or when applying de novo identification, these proteins can be identified.*

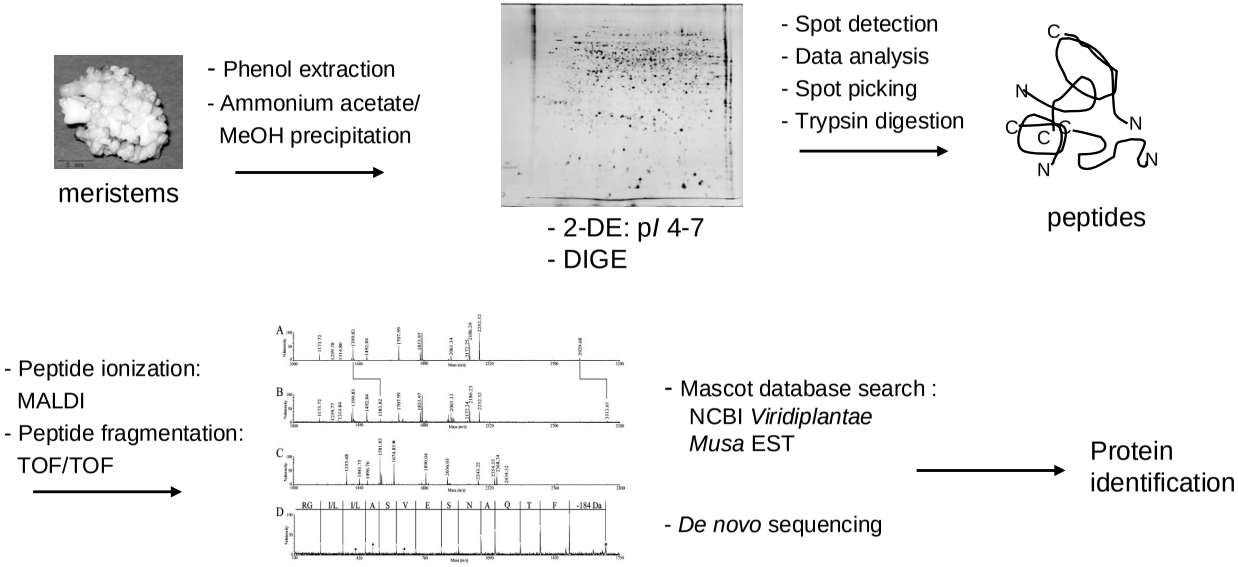

Figure 1.6: *A schematic overview of the workflow followed in a banana proteomics experiment*

Chapter 2

Membrane proteomics

2.1 Introduction

A fascinating subgroup of proteins is the group of the membrane proteins. These proteins are involved in many important cellular functions such as transport of small molecules through the membrane, cell-cell interactions, recognition of changes in environmental conditions and generating the appropriate response. Two main categories of membrane proteins can be distinguished; those that span the membrane (i.e., integral membrane proteins (IMP)) (figure 2.1A) and those that are associated with the membrane lipid layer or with the integral membrane proteins (i.e., peripheral membrane proteins) (figure 2.1B) (Tan et al., 2008). Integral transmembrane proteins are generally classified as monotopic (figure 2.1A4), bitopic (figure 2.1A1) or polytopic (figure 2.1A2). Monotopic proteins are located at only one side of the membrane. The proteins that traverse the membrane one time (i.e., bitopic) are further divided into type 1 and type 2 IMP. Type 1 proteins are oriented with their amino-terminus facing the luminal or extracellular space, while type 2 proteins have the opposite orientation (Tan et al., 2008). The IMP proteins of type 1 can be further divided into proteins with (type1a) and without (type1b) cleavable signal sequences. Peripheral membrane proteins are attached to the membrane either indirectly by interactions with integral membrane proteins (figure 2.1A7 and 8), either directly by interactions with the lipid polar head groups (figure 2.1A5 and 6).

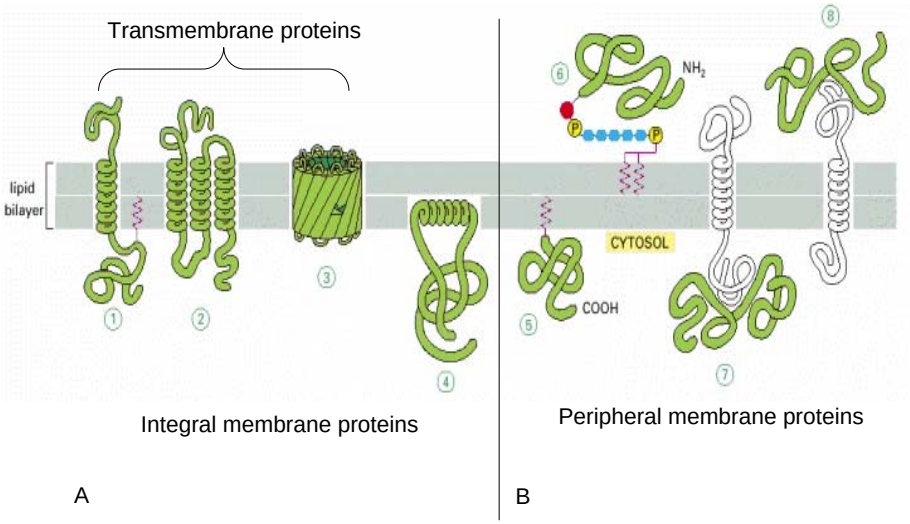

Figure 2.1: *Membrane proteins are generally classified as integral (left) or peripheral (right). Most IMP cross the bilayer as a single α helix (=bitopic, 1), as multiple α helices (=polytopic, 2), or as a rolled-up β sheet (a β barrel) (3). Some IMP are only located at one side of the membrane (=monotopic, 4). Peripheral membrane proteins are covalently attached to membrane lipids (5) or by an oligosacaccharide anchor (6). Others are attached to the membrane by interactions with IMP (7, 8). Adapted from Alberts et al. (2002), p.594*

One of the most common lipid anchors is a glycosyl-phosphatidylinositol (GPI) anchor (figure 2.1A6). The GPI-anchored proteins are generally located at the extracellular or luminar part of the membrane. The other peripheral membrane proteins are attached to the cytosolic face of membranes by a hydrocarbon moiety (e.g., a prenyl, farnesyl, or geranylgeranyl group) at the carboxy-terminus or by a fatty acyl group (e.g., myristate or palmitate) at the amino-terminus (figure 2.1A5). These proteins are generally located at the cytosolic part of the membrane.

Although IMP represent 20 (e.g. in *Arabidopsis thaliana*) to 40% (e.g. in *Plasmodium falciparum*) of all open reading frames (Krogh et al., 2001), they are highly underrepresented in most classical proteomic studies.
The first paper dealing with a proteomics analysis of membrane proteins using 2-DE was published in 1976 by Ames and Nikaido (1976). Since no reliable protein identification was performed, there was no certainty about the real nature of the proteins on the gel. With the development of high-throughput

proteomics and mass spectrometric identification techniques, it was demonstrated that classical 2-DE maps hardly display any membrane protein (Adessi et al., 1997; Wilkins et al., 1998). Only outer membrane proteins of Gram-bacteria were easily separated using 2-DE (Molloy et al., 2001) because they span the membrane with beta barrels. These barrels are closed β-sheets which consist of a number of antiparallel β-strands (figure 2.2A) and are composed of an alternation of hydrophobic and hydrophilic amino acids. The hydrophobic amino acids are oriented to the lipid bilayer and the hydrophilic amino acids to the inside, as such forming a pore (Alberts et al., 2002). Because of this amino acid alteration, there is no long stretch of hydrophobic amino acids and the protein can easily be solubilized in aqueous buffers.

Most other IMP span the membrane by α helices. These helices consist of long (15-25 AA) stretches of hydrophobic amino acids, which are coiled into a helix (figure 2.2B). The interaction of these helices with the lipids of the membrane is stronger compared to beta barrel proteins and a strong detergent is thus required to release the proteins from the membrane. Unfortunately, strong detergents are not compatible with the first dimension iso-electric focusing step of classical 2-DE.

Next to the solubility problem, another reason why IMP are frequently missed in proteome studies is that membrane proteins are generally less abundant compared to soluble proteins hindering their detection.

In this chapter, solutions to solve these two problems are discussed. First, suggestions to solve the abundance (section 2.2) and solubility (section 2.3) problem are listed, followed by an overview of methods to study membrane protein complexes (section 2.4). Subsequently, methods to evaluate the number of membrane proteins identified in a proteomics experiment are discussed (section 2.7). Finally, some applications in plants will be discussed (section 2.8).

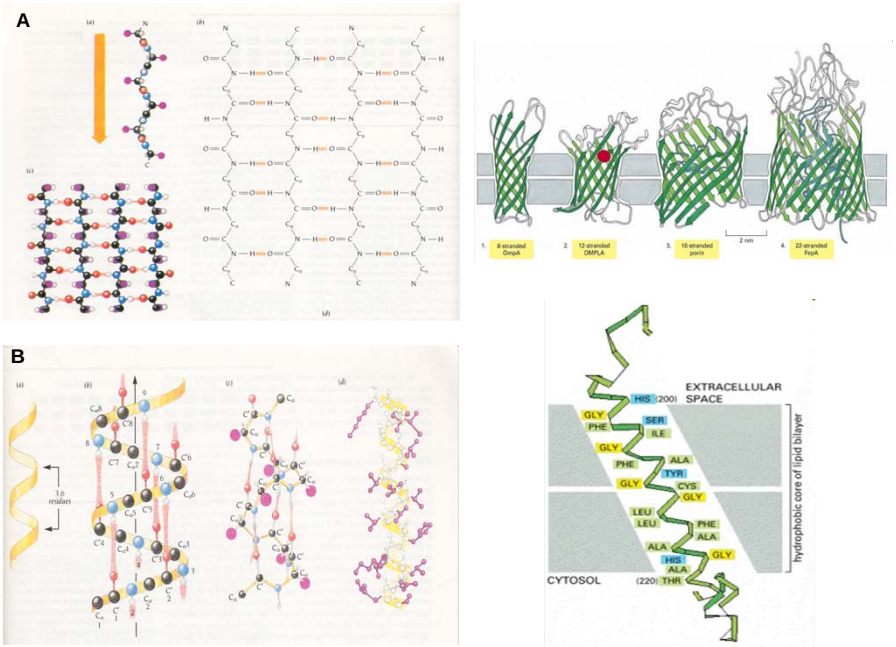

Figure 2.2: *Different representations of beta sheets (A) and alpha helices (B) adapted from* `http://tigger.uic.edu` *(left) and the way they are spanning the membrane (right; adapted from Alberts et al. (2002), p.596,598)*

2.2 The abundance problem: solutions

The best solution to handle the abundance problem is to enrich the original sample in membrane proteins. Several pre-fractionation techniques have been developed, based on the physico-chemical or biochemical properties of membrane proteins. The most common enrichment strategy is subcellular fractionation combined with removal of non-membrane and membrane-associated proteins (Ephritikhine et al., 2004). An alternative to this strategy is phase partitioning. Peltier and his colleagues describe a three phase partitioning with 1-butanol (Peltier et al., 2004). Everberg and co-workers optimized a detergent/polymer two phase partitioning system to facilitate the study of membrane proteins of yeast mitochondria (Everberg et al., 2004, 2006).

2.2.1 Subcellular fractionation

Subcellular fractionation is generally performed in steps. The first step is the preparation of microsomes or of a total membrane fraction. When a cell is lysed in an aqueous, detergent-free medium, membranes fragment into vesicles. These vesicles are subsequently separated from the bulk of soluble proteins through sedimentation (e.g., ultracentrifugation). Subsequently, most researchers focus on a specific organelle. A common approach to isolate the organelle of interest is density gradient centrifugation. Percoll gradients are used for the isolation of plastids or mitochondria and sucrose or sorbitol gradients for endoplasmatic reticulum (ER) and tonoplast (Ephritikhine et al., 2004). Sometimes free flow electrophoresis is used as an alternative technique (Heidrich and Hannig, 1989; Braun et al., 2007).

2.2.1.1 Focus on the plasma membrane

Since the plasma membrane (PM) is located at the interface between the cell and the environment, PM proteins are involved in sensing changes in this environment and generating the appropriate defense response. Several techniques were developed for the isolation of this interesting membrane. Valot and his colleagues applied sucrose gradient centrifugation to isolate plasma membranes of *Medicago truncatula* in a study of arbuscular mycorrhiza-related proteins in root plasma membrane fractions (Valot et al., 2005).

A more widely applied technique is aqueous-polymer two-phase partitioning (Widell et al., 1982). Most aqueous mixtures of two structurally different water soluble polymers give rise to two phases when their concentration is above a certain value (i.e., the critical concentration). The most widely used system for plasma membrane isolation consists of a mixture of polyethylene glycol (PEG) and dextran (Schindler et al., 2006). Since the lipid composition of plasma membranes differs from that of endomembranes, both membrane types have a different affinity for both polymers. Plasma membranes will preferentially partition into the hydrophobic PEG upper phase, while endomembranes reside in the dextran lower phase. However, also a portion of endomembranes will be present in the PEG upper phase. To obtain a purer PM fraction, subsequent rounds of phase partitioning are performed (see chapter 5). Also the use of affinity ligand wheat germ agglutinin (WGA) coupled to dextran is reported

to further purify the PM fraction (Persson and Jergil, 1994). This WGA attaches to glycoproteins associated with the plasma membrane and pulls plasma membranes into a dextran-enriched phase.

A third method to isolate plasma membranes is the cationic colloidal silica technique (Chaney and Jacobson, 1983). In this method the extracellular side of the plasma membrane is coated with silica particles through interactions between the anionic phospholipid head groups and sialic acid groups of glycoproteins with positively charged silica. This coating enhances the density of the plasma membrane which makes isolation by density centrifugation possible.

An alternative method uses the extracellular domains of IMP for affinity purification. Two commonly used affinity purification techniques are biotinylation and glycosylation affinity purification. In the first method, reviewed by Elia (2008), plasma membrane proteins are selectively labeled with a biotinylation reagent that can not penetrate cells. After cell lysis, cells are passed over avidin or streptavidin affinity columns. The labeled proteins will retain on the column, thereby isolating plasma membranes from the rest of the cell content. Glycosylated proteins can be isolated using lectin-mediated affinity chromatography (Ghosh et al., 2004). However, this method is more suited for the analysis of specific glycoproteins instead of total plasma membrane fractions.

Also the use of magnetic beads with immobilized antibodies against plasma membrane proteins has been proposed as an alternative technique for the isolation of plasma membranes (Lawson et al., 2006).

Since plant cells are surrounded by a protecting cell wall, methods relying on interactions with plasma membrane proteins such as the affinity based purifications, are not applicable to intact plant cells. Plant researchers therefore choose for a sucrose gradient or two phase partitioning for isolation of plasma membranes. Another possibility could be to first prepare plant protoplasts by an enzymatic digest (i.e., a combination of cellulase, pectinase and hemicellulase) of the cell wall.

Lipid rafts A special feature of plasma membranes is the presence of lipid rafts. These are functional microdomains in membranes, formed by a clustering of sphingolipids and sterols which are insoluble in nonionic detergents (e.g. Triton X-100) at 4 ℃ (Brown and London, 2000). As such, proteins associated with these domains can easily be isolated by collecting the detergent resistant

fraction. These proteins play important roles in protein sorting, signal transduction, cell wall metabolism and responses to biotic and abiotic types of stress (Morel et al., 2006).

2.2.2 Enrichment

After purification of the membrane structure, contaminating compounds such as lipids, membrane-associated proteins and soluble proteins that are trapped in the membrane vesicles are still present. Several techniques were developed to remove them.

2.2.2.1 Removal of membrane-associated proteins

One way to remove soluble proteins that are trapped inside a membrane vesicle is through sonication. During sonication, vesicles are broken and reformed thereby releasing the soluble proteins (Ephritikhine et al., 2004). An alternative is performing a Brij58 wash (see also chapter 5) which turns the vesicles inside out (Behzadipour et al., 2001).

If the goal is to study IMP, membrane associated proteins can be removed by washing the membranes with high salt and/or high pH buffers (Fujiki et al., 1982; Howell and Palade, 1982; Pasquali et al., 1997). A high salt wash disrupts electrostatic interactions between the phospholipid bilayer and proteins and between peripheral and integral membrane proteins. At high pH (≥ 11), membranes do not reseal after mechanical agitation but form membrane "sheets" which are the result of fusion of vesicles followed by their rupture (Howell and Palade, 1982). As such, trapped soluble proteins are released. Integral membrane proteins are usually only soluble in detergents or organic solvents and will not be affected by these washes.

2.2.2.2 Enrichment in hydrophobic proteins

Membrane fractions can be enriched in hydrophobic proteins through sequential solubilization of proteins (Molloy et al., 1998; Lehner et al., 2003). In this approach, hydrophobic proteins are separated from the hydrophilic ones by increasing the concentration of chaotropes (urea and thiourea) and/or detergent.

A very stringent method to enrich in hydrophobic proteins is protein extraction in a mixture of chloroform and methanol (Joyard et al., 1982) which will be further explained in chapter 3.

None of these methods, however, is sufficiently stringent to remove all soluble contaminants (Ephritikhine et al., 2004; Schindler et al., 2006; Rabilloud et al., 2008).

2.2.3 Delipidation

Also lipids have to be removed from the membrane fraction because they will interfere with protein separation and MS analysis (Speers and Wu, 2007). A widely used method is chloroform/methanol precipitation (Wessel and Flugge, 1984). Lipids are extracted into the chloroform fraction while proteins precipitate at the chloroform-methanol interface. However, as stated above and demonstrated in chapter 3, dependent on the C/M ratio, some proteins will remain soluble in the chloroform phase.

Lipids can also be removed through protein precipitation with di-ethylether or acetone.

2.3 The solubility problem: solutions

After isolation of the membrane structure of interest which offers a solution to the abundance problem, membrane proteins need to be released from the membranes and kept in solution during the complete analysis process. When membrane proteins are released from their natural hydrophobic environment and are solubilized in an aqueous solution there is a high risk on protein aggregation and precipitation. Several solutions to cope with this solubility problem have been proposed and are listed below.
There are more problems associated with the solubility of membrane proteins in the gel-based approach compared to the gel-free approach since gel-free methods can circumvent some of the solubility problems by focusing on soluble peptides (i.e., peptides originating from the outer-or luminal parts of the protein).

2.3.1 Solutions in the gel-free approach

2.3.1.1 Protein solubilization: use of alternative detergents

Optimal protein digestion is crucial to obtain good results in a gel-free approach. Therefore, the reagents used for IMP solubilization need to be compatible with the used protease, often trypsin (see chapter 1, section 1.6). A strong detergent such as SDS would be the first choice but high concentrations of this detergent inhibit trypsin activity. Moreover, SDS suppresses peptide ionization and interferes with chromatographic separation (Beavis and Chait, 1990). Therefore, it has to be removed or diluted (Puchades et al., 1999) before protein digestion. Two different approaches are applied. The first approach uses in-gel digestion to remove SDS and other interfering substances during washing. Different alternatives for in-gel digestion have been suggested. Proteins can be separated using SDS-PAGE and bands with proteins can subsequently be excised. When protein separation is not desired (e.g., to shorten the sample processing time), in-tube gel digestion (Lu and Zhu, 2005) or gel-assisted digestion (Lu et al., 2008b) is performed. An alternative is to stop protein migration when proteins have entered the separating gel and to excise the complete stacked protein band in one time (e.g., Marmagne et al. (2007)). In the second approach, SDS is exchanged by urea aided by a filter. This filter assisted sample preparation is described by Wisniewski et al. (2009).

Another approach is to search for alternatives of SDS. A first alternative is the use of acid-labile surfactants (ALS) at physiological pH (Yu et al., 2003). After protein digestion, pH is lowered for MS analysis. At this low pH the ALS are hydrolyzed and precipitate. As such, interference with reversed-phase separation and ionization is minimized. Currently, two commercially ALS are available; RapiGest (Waters) and 3-[3-(1,1-bisalkyloxyethyl)pyridin-1-yl]propane-1-sulfonate (PPS, Protein Discovery). In case sample preparation requires an acid pH or the analyte of interest is acid labile, a non-acid cleavable detergent can be used (Norris et al., 2005).

A disadvantage of cleavable detergents is the co-precipitation of hydrophobic proteins together with the degradation products. Therefore, the commercially available, mass spectrometry compatible detergent Invitrosol (Invitrogen) has been developed. The detergent elutes in three peaks that are well separated from the elution times of most peptides and does thus not interfere with protein identification.

2.3.1.2 Protein digestion

Since a good protein digestion is of utmost importance, also adaptations to facilitate protein digestion of membrane proteins have been suggested.

A. Organic solvents A first option is performing the digestion in the presence of organic solvents because they facilitate tryptic digestion of membrane proteins (Russell et al., 2001; Blonder et al., 2002; Strader et al., 2006; Mitra et al., 2009). Probably the solvents serve as denaturants during digestion. An advantage of the use of organic solvents is that they can easily be removed prior to MS analysis. Since the solubilization capacity of organic solvents is limited, they are frequently combined with other techniques. Chen and coworkers demonstrated that combining 80% acetonitrile with RapiGest or PPS results in a large increase in the number of peptide identifications (Chen et al., 2007a). We used a methanol-assisted protein digestion to study plasma membrane proteins from banana leaves (chapter 5).

B. Alternative proteases Since the trypsin cleaving targets, the hydrophilic amino acids R and K, are hardly present in transmembrane domains consisting of alpha helices, a tryptic digest often results in the identification of peptides from the soluble parts of the protein. To ensure protein cleavage in the transmembrane domains (TMD), other proteases than trypsin are applied, alone or in combination with trypsin. One example is chymotrypsin which cleaves peptide bonds of amino acids with aromatic or large hydrophobic side chains such as Y, W, F and M (Fischer et al., 2006). Another possibility is the use of cyanogen bromide (CNBr) which cleaves after M in the presence of a strong acid (e.g., 90% formic acid) (Washburn et al., 2001). In the filter-assisted sample preparation method, Lys-C is used prior to trypsin since this protease allows digestion in the presence of high urea concentrations (Wisniewski et al., 2009).

Trypsin can also be replaced by proteinase K (pK), a nonspecific protease (Wu et al., 2003). This method takes advantage of the high pH conditions that are frequently used to remove soluble proteins from membranes (see 2.2.2.1). After removing the soluble proteins, pK is added to the membrane sheets to "shave off" the soluble domains of IMP. Recently the group of Karas demonstrated the value of elastase (Rietschel et al., 2009a) and pepsin (Rietschel et al., 2009b) for the digestion of membrane proteins.

2.3.1.3 Improved separation strategies

To further improve the success rate of IMP identification, adaptations of the chromatographic separation step have been suggested. One possibility is the use of an extended long (60-65)μ-LC column under high pressure (Motoyama et al., 2006). Liu and her colleagues applied a RP step gradient from 10% to 30% ACN to the SCX column in the presence of a high salt concentration to recover hydrophobic peptides that were not released from the SCX column (Liu et al., 2006). Another alternative is to conduct the μ-LC separation of enriched transmembrane peptides at elevated temperature (Xiang et al., 2006; Speers et al., 2007). An overview of the discussed enrichment strategies with their merits and drawbacks is given in table 2.1.

Table 2.1: *Merits and drawbacks of the techniques used to improve the detection of membrane proteins*

Technique	Pro	Contra
Prefractionation[1]		
1. Centrifugation	no specialized equipment except when continuous gradients used	multiple steps no pure fractions
2. Phase partitioning	one step no specialized equipment	no pure fractions
3. Immunoaffinity purification	repeated purifications from one sample resulting in high yield	high costs long procedure complex samples need prior fractionation step
4. Free flow electrophoresis	one step high sample recovery intact organelles	specialized equipment cellular strucures with similar p*I* comigrate
Removal contaminants[2]		
1. Sonication	fast and easy	only small amount contaminants removed
2. Brij 58	all vesicles have same orientation	only trapped soluble proteins removed
3. Differential solubilization	purer fractions	multiple steps (protein losses)
4. Salt wash	removal peripheral proteins	not so efficient
5. High pH	compatible with proteinase K	removal not complete
6. Organic solvents	reduces sample complexity	highly selective
7. Delipidation	no lipid interference during MS analyses	protein losses
Protein digestion[3]		
1. In presence of organic solvents	improved tryptic cleavage	transmembrane regions without R or K not cleaved
2. Alternative proteases	transmembrane regions are cleaved	cleavage sites often not predictable

[1] a.o., based on Lee et al. (2010)

[2] a.o., based on Ephritikhine et al. (2004)

[3] a.o., based on Lu et al. (2008a)

2.3.2 Solutions in the gel-based approach

As stated above, gel-free techniques show the least problems in the identification of membrane proteins, provided these proteins are sufficiently abundant and are thus the most powerful solution to study IMP from well characterized species. As demonstrated on figure 1.5 in the previous chapter, characterized species are preferably studied through a gel- based approach with sufficient resolving power. Therefore, adaptations to the classical 2-DE protocol are proposed to make 2-DE compatible with the separation of IMP. Additionally, alternative gel-based protocols for membrane proteomics have been developed.

2.3.2.1 Adaptations to classical 2-DE

Since no technique has a better resolving power than classical 2-DE, researchers tried to make it suitable for the study of membrane proteins. Solubility of membrane proteins is troublesome at three points during a classical 2-DE protocol.

A first problem is the initial protein extraction and solubilization. Since the use of SDS is excluded because separation in IPG strips during IEF requires low ion concentrations, other, often less powerful detergents need to be used. Nonionic or zwitterionic detergents such as ASB 14 (Chevallet et al., 1998; Rabilloud et al., 1999), dodecyl maltoside, Triton X-100 and Brij 56 (Luche et al., 2003) have therefore been proposed.

Once applied on a IPG strip, a second problem is encoutered when hydrophobic proteins precipitate at their p*I*. This precipitation was reported and demonstrated by several authors such as Coughenour et al. (2004) and Klein et al. (2005). Adessi et al. (1997) describe the interaction of hydrophobic proteins with the IPG acrylamide-matrix at or close to their isoelectric point. Some researchers tried to reduce this aggregation by using soft IPG-strips with larger polyacrylamide pores (Candiano et al., 2002), others use agarose in stead of polyacrylamide (Altenhofer et al., 2006).

A third problem occurs during the transfer from the first to the second dimension. Eravci and his colleagues report the accumulation of membrane proteins at the top of the second dimension gel (Eravci et al., 2008).

Despite the efforts made to improve the 2-DE protocol, the separation of IMP with more than two membrane spanning regions remains troublesome. A modified 2-DE protocol is a good solution for the separation of membrane-associated

proteins and membrane proteins with minor hydrophobicity but highly hydrophobic membrane proteins require alternative gel-based methods that avoid the use of IEF.

2.3.2.2 Alternatives for classical 2-DE

A. One dimensional electrophoresis A first approach is to skip IEF and to apply only one dimensional SDS-PAGE. However, 1D-SDS-PAGE only separates protein samples into a limited amount of individual protein bands (Williams et al., 2006). Therefore, this method requires a stringent prefractionation step (e.g., as described in chapter 3). When separation of protein samples with higher complexity is required, an alternative two dimensional electrophoresis protocol is required.

B. Double SDS-PAGE A first and logical adaptation to 2-DE is the use of SDS-PAGE in both dimensions since in this way the strong detergent SDS is utilized for protein solubilization in both dimensions. The base for the development of SDS/SDS- PAGE (dSDS-PAGE) by Akiyama and Ito (1985) was the observation that hydrophobic proteins can bind up to 3 gram SDS per gram protein (Buxbaum, 2003) in stead of 1.5-2 g. Rath et al. (2009) even report the binding of 10g SDS/g protein for some membrane proteins. Due to the higher amount of bound SDS, membrane proteins show anomalous migration during SDS-PAGE. However, this effect only occurs when pore size is not limiting. In a highly concentrated gel with small acrylamide pores the molecular sieving effect predominates and hydrophobic and hydrophilic proteins of the same size migrate at the same speed (Beyreuther et al., 1980). When combining gels with different pore sizes in both dimensions, membrane proteins can be separated from hydrophilic proteins with a normal migration pattern (figure 2.3).

After a first separation, a lane of the first dimension gel with large pores ($\leq$ 10 % acrylamide) is excised and laid on top of a second dimension gel with narrow pores ($\geq$ 15 % acrylamide). This results in protein spots which are dispersed around a diagonal. Recently, it has been demonstrated that the principle of the anomalous migration of membrane proteins is not readily explained because membrane proteins will only bind such a high amount of SDS when they are completely denaturated (Rath et al., 2009). However, the aberrant migration pattern was demonstrated for all membrane proteins (Rais et al., 2004).

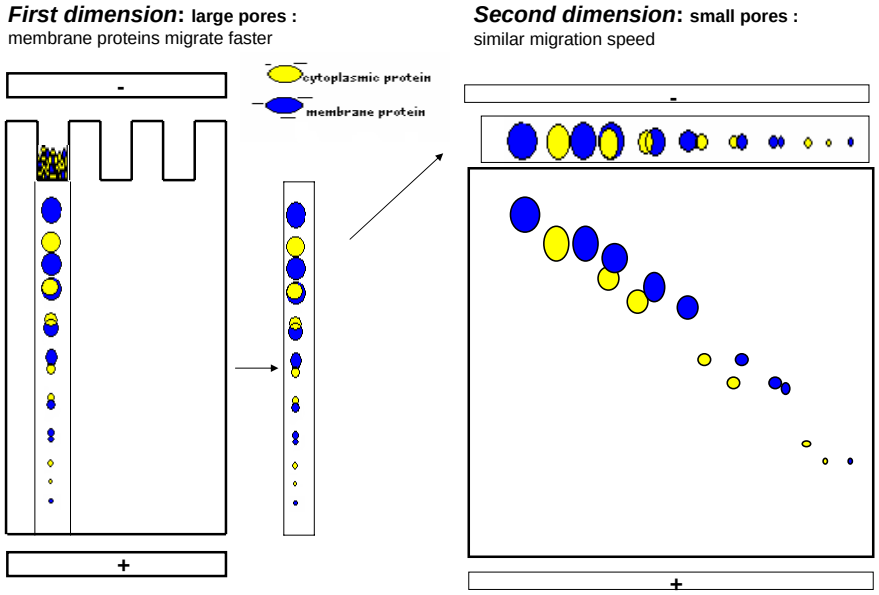

Figure 2.3: *Principle of dSDS-PAGE. Proteins are separated in the first dimension through SDS-PAGE on a gel with large pores. Due to a stronger negative charge, membrane proteins will migrate faster than soluble proteins. In the second dimension SDS-PAGE, gel pores are smaller and all proteins of the same molecular size migrate at the same speed.*

Since in the dSDS technique SDS is used as solubilizing agent in both dimensions, resolution remains limited. It can be slightly improved, especially for low molecular weight proteins, by using Tricine SDS-PAGE and by addition of urea to the first dimension gel (Rais et al., 2004). Further optimization by Williams et al. (2006) comprised the incorporation of glycerol and increased Tris concentrations into the gel solutions and the use of Bicine instead of Tricine as trailing ion. Despite these optimizations, resolving power remained limited. Therefore alternatives were sought in techniques that replace SDS in one dimension by another detergent.

C. Cationic electrophoresis Resolution of a two dimensional separation increases in case different situations are used in the two dimensions. Since SDS is preferentially used in one dimension, the detergent used in the other dimension

needs to display a different behavior. Therefore, the use of cationic detergents is an obvious choice. In 1971, Williams and co-workers demonstrated the power of the cationic detergent cetyltrimethylammoniumbromide (CTAB) (Williams and Gratzer, 1971). Penin et al. (1984) suggested to use this detergent for membrane proteome studies. Because results were very promising, other cationic detergents were tested as well. Benzyldimethyl-n-hexadecylammonium chloride (16-BAC) proved to be a valuable alternative (Macfarlane, 1989; Hartinger et al., 1996). When added above their critical micellar concentration, CTAB (Eley et al., 1979) and 16-BAC (Macfarlane, 1983) bind at a constant ratio to proteins, analogous to SDS. They are able to mask the intrinsic charge of proteins by giving them a positive charge. Consequently, detergent-protein complexes migrate towards the cathode and require a different buffer (Jovin, 1973) and gel system compared to SDS-PAGE. Gel polymerization is initiated by addition of H_2O_2 which causes a less stable polymerization compared to the Temed, ammoniumpersulfate-based acrylamide polymerization. Gradually, systems were optimized to improve stacking of proteins and enhance resolution (Buxbaum, 2003; Zahedi et al., 2005; Kramer, 2006). However, separation in both dimensions still relies on differences in molecular size. As a result, protein separation results in a dispersed diagonal. Although resolution was improved compared to dSDS, it was still not comparable with the resolution of classical 2-DE. Zahedi and colleagues proposed the use of tube gels for the first dimension separation to improve transfer to the second dimension and to improve resolution (Zahedi et al., 2005), but again the result was not convincing. Since its first publications this method remained largely unnoticed. Only the last years this method started to gain popularity. In 2007, Braun and his colleagues reviewed the most important papers (Braun et al., 2007).

D. Other techniques Schluesener and colleagues replaced the first dimension by anion-exchange chromatography for analysis of the membrane proteome of *Corynebacterium glutamicum* (Schluesener et al., 2005). They report membrane proteins with one up to 13 transmembrane helices and describe coverage of proteins from a wide pH (pI 3.7-10.6) and mass range (10-120 kDa). Another approach deals with the detection of membrane proteins in gel (Hart et al., 2004). The authors use a fluorescence-based staining technique for visualization of integral membrane proteins developed by Molecular Probes, Inc (Eugene, OR). The researchers state that the Pro-Q Amber gel stain selectively

highlights proteins containing two or more α helical transmembrane domains. However, these techniques are less widespread.

2.4 Protein-protein interactions

The generation of a physiological response requires that several proteins interact with each other. Knowledge of the composition and structure of protein complexes in the cell is important to improve the understanding of cellular processes and pathways. Membrane proteins often play crucial roles in physiological processes as a starting point of a reaction or as a point where signals are transferred from the cytoplasm to a specific cellular compartment. A special challenge in the study of complexes composed of membrane and membrane-associated proteins is to avoid disruption of the complex when disturbing the lipid bilayer.

Most existing methods are developed to study interaction partners of known (membrane) proteins. These methods include two-hybrid methods (Fields and Sternglanz, 1994; Fields, 2009), affinity purification techniques (e.g., co-immunoprecipitation (Poetz et al., 2009) and tandem affinity purification (Gingras et al., 2007)), fluorescence resonance energy transfer (Clegg, 1996) flow cytometry (Dye et al., 2005), surface plasmon resonance (Kim et al., 2005), site-directed mutagenesis (Stephens et al., 2003), protein correlation profiling (Andersen et al., 2003) and NMR and X-ray crystallography (Burz et al., 2006).

Sometimes a general overview of all protein complexes in a cellular or subcellular fraction is desired without previous knowledge of a bait protein. In that case native electrophoresis is the method of choice.

When using native electrophoresis, proteins and protein complexes need to remain in their native state during their isolation. Therefore, no denaturating agentia are added to the extraction buffer and all steps are performed under cold conditions. In plants, the technique is mainly applied to study mitochondrial or chloroplast complexes (Eubel et al., 2005), using isolated organelles as starting material but also cytoplasmic complexes have been studied (Remmerie et al., 2009). Three different variants of the same basic technique are applied; clear native electrophoresis (CN), blue native electrophoresis (BN),

and high resolution clear native electrophoresis (hrCN) (Krause, 2006; Wittig and Schägger, 2008). They differ in the composition of the cathodic buffer. In CN, protein complex separation is based on the intrinsic charge of the proteins that build the protein complex. This charge depends on the pH of the cathodic buffer. Proteins having a pI lower than the pH of the buffer are negatively charged and migrate towards the anode. Proteins having a pI>pH of the buffer are positively charged and will be lost for analysis. No solubilizing agentia are added during extraction and separation. This makes clear native electrophoresis less suitable for the analysis of membrane protein complexes because these will aggregate. A better option is BN where the negatively charged protein-binding Dye Coomassie Brilliant Blue G-250 is added to the cathode buffer.

This renders all complexes with a negative (and as such repelling) charge. However, several basic soluble proteins do not bind this dye and will be lost during analysis. In hrCN mixed anionic/neutral detergent micelles are added to the cathode buffer which also provide a negative charge to the complexes. The most robust technique of these three is BN and is therefore often preferred (Wittig and Schägger, 2009).

Blue native electrophoresis was pioneered by Schägger and von Jagow to separate mitochondrial membrane proteins and complexes (Schägger and von Jagow, 1991; Schägger et al., 1994). A very detailed description of the BN protocol is given by Reisinger and Eichacker (2006, 2008).

The first step in the BN protocol is the isolation of the complex in its native state, i.e., with all its subunits. These complexes can be iolated from total cellular lysates, but generally purified membrane fractions are used. The success of complex solubilization depends on the pH, ionic strength and the detergent used in the extraction buffer. The pH needs to be close to the physiological pH to retain the complexes in their native state. High salt concentrations are deleterious for complex isolation since they distort protein-protein interactions. Therefore, the salt concentration has to be kept low (i.e., 50 mM NaCl). An alternative is the use of aminocaproic acid. This zwitterionic compound supports complex solubilization similar to NaCl, but does not disturb protein-protein interactions at higher concentrations and can be used safely in the extraction buffer (Reisinger and Eichacker, 2006). Good detergents leave some specific lipids bound to the membrane proteins to keep complexes intact. Generally applied detergents in plant research are Triton X-100, digitonin and

n-dodecyl-b-D-maltoside (Eubel et al., 2003, 2005). Digitonin is the mildest of the three and is preferred when studying supercomplexes, which are associations of single complexes (Heinemeyer et al., 2004; Strecker et al., 2010). We will also use it in a study of banana membrane protein complexes (see chapter 4). Since optimum detergent conditions are complex-specific, these are ideally determined for every complex under investigation. However, to compare different complexes in the same cellular or subcellular fraction, standard solubilizing conditions are required. Generally, mild neutral detergents are used in a detergent/protein ratio around 2-3 g/g protein to avoid extensive delipidation and denaturation.

After extraction and solubilization, protein complexes are separated according to their size in a native gradient gel. A large difference in pore size from the top to the bottom of the gel (e.g., 4-16 % acrylamide) is required to cope with the large difference in M_r between the biggest and smallest complex. To provide uniform separation, CBB is added to the solubilization and cathodic buffer because the dye provides all complexes with a negative charge. An additional advantage is that the complexes are visualized during separation.

The stained bands on this first dimension gel can be excised and proteins present in one band (and as such belonging to the same complex) can be identified. It is also possible to visualize the individual subunits of one complex by denaturation of the complex and subsequent separation of the individual subunits. For second dimension separation, generally SDS-PAGE is utilized. This BNE strategy is demonstrated in figure 2.4.

After the first dimension separation, gel lanes containing the separated complexes are excised and equilibrated in a buffer containing SDS. The strong detergent decomposes the complexes and the individual subunits are subsequently separated using a normal SDS-PAGE. To improve resolution, three dimensional electrophoresis can be performed, combining 2 variants of native electrophoresis in the first and second dimension and SDS-PAGE in the third dimension (Wittig and Schägger, 2009). Other adaptations combine BN with continuous elution electrophoresis (Huang et al., 2009), LC-MS/MS (Fandino et al., 2005; Katz et al., 2007) or classical 2-DE (Werhahn and Braun, 2002).

An overview of all alternative gel-based techniques for membrane proteomics and their performance in comparison to a gel-free (2-D LC) approach, can be found in table 2.2.

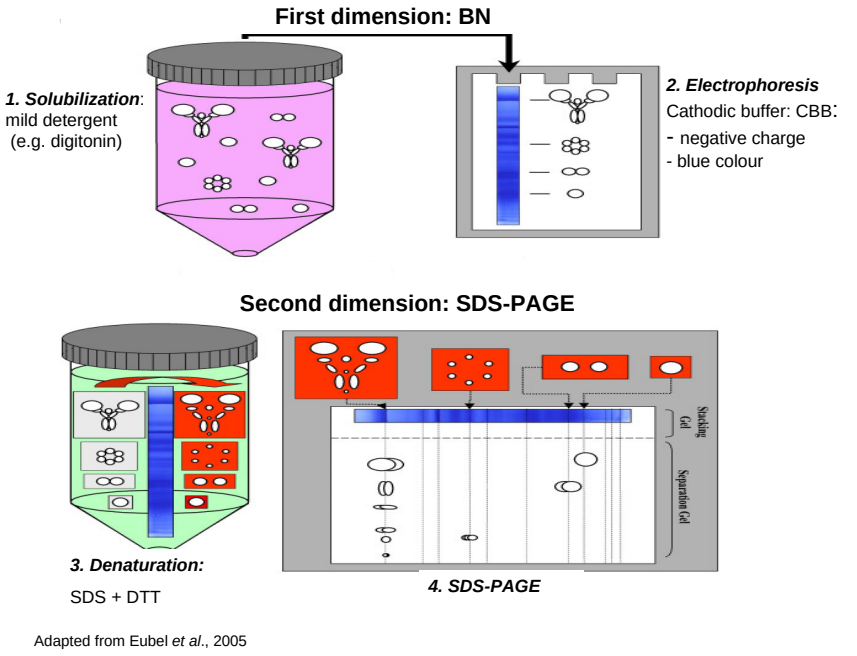

Figure 2.4: *Protein complexes are first solubilized using a mild, non-ionic detergent and separated through native electrophoresis. The CBB dye is added to the cathodic buffer to provide sufficient negative charges and to visualize protein separation. When native electrophoresis is terminated, gel lanes containing the separated complexes are excised and denaturing agentia are added. Finally the individual complexes are separated through SDS-PAGE. Proteins belonging to the same complex or proteins that migrated to the same distance in the one dimensional gel will be located on the same vertical line.*

Table 2.2: *Comparison of the existing techniques for membrane proteomics. Different criteria are evaluated and scored based on a.o., Braun et al. (2007), Rabilloud et al. (2008) and personal experiences. The score ranges from +++ (excellent) to - - - (very poor).*

Criterion	**Modified 2-DE**	**1-D SDS-PAGE**	**dSDS-PAGE**	**Cationic**	**BN-PAGE**	**GeLC**	**2-D LC**
Analysis IMP	- - -	+ + +	+ + +	+	+	+ +	+ +
Resolution	+ + +	- - -	- -	-	+	+ +	+ +
Reproducibility	+ + +	+	+	+	+	+	-
Usability	+ +	+ + +	+	-	+	+ +	+ +
Quantitative studies	+ +	- - -	- -	-	-	+ +	+
Detection PTM	+ + +	- - -	- -	- -	-	-	-

2.5 Quantification

Problems with protein quantification are mainly observed in the gel-based approaches that have been adapted for membrane proteomics. Since all current gel-based methods use a separation according to molecular size in both directions, resolution remains limited for all existing techniques. This implies that protein spots on these gels generally contain more than one protein which makes quantification troublesome. Moreover, the two-dimensional gel-based techniques described in this chapter rely on excision of gel lanes from the first dimension gel which are subsequently transferred to the top of the second dimension gel. This makes the method less reproducible and makes a quantitative comparison between gels unreliable in comparison to classical 2-DE. By labeling with the fluorescent Cydyes (see section 1.4 of chapter 1), this problem can partly be circumvented since at least two samples will undergo the same process, as demonstrated by Heinemeyer et al. (2009).

2.6 A combination of techniques will provide the best results

Since a proteome is always very diverse, none of the existing techniques will be able to analyze the complete membrane proteome at once. Different authors therefore propose the use of a combination of techniques to obtain the most complete picture. By using different fractionations based on the physicochemical properties of proteins and one dimensional electrophoresis, Brugière et al. (2004) were able to identify 44 previously unidentified mitochondrial membrane proteins from *Arabidopsis* cell suspensions. Friso and co-authors combined one- and two-dimensional electrophoresis to analyze the thylakoid membrane proteome of *Arabidopsis thaliana* chloroplasts (Friso et al., 2004). Coughenour combined classical 2-DE with 16-BAC to analyze a synaptic vesicle proteome (Coughenour et al., 2004). By combining 1-D SDS-PAGE, 16-BAC/SDS-PAGE and dSDS-PAGE, Burré et al. (2006) increased the number of identified hydrophobic proteins in these vesicles. Zahedi and his colleagues recommend the combination of dSDS and 16-BAC for the analysis of membrane proteins (Zahedi et al., 2005).

In conclusion, proteins with minor hydrophobicity can be separated by a classical 2-DE protocol by changing the detergents used. Best results are obtained when prefractionated samples are analyzed. Specific enrichments like chloroform/ methanol extraction can highly reduce the complexity of samples so one dimensional separation can give sufficient resolution to separate and identify individual proteins. For a broader view on membrane proteins, SDS/SDS and 16-BAC/SDS or CTAB/SDS-PAGE can be applied. Although 16-BAC/SDS-PAGE (or CTAB-SDS-PAGE) provide a higher resolution than dSDS-PAGE, the latter method has proven to separate more highly hydrophobic proteins (Burré et al., 2006). Probably the strong solubilizing power of SDS is required. By applying dSDS, the authors could analyze 28 integral synaptic vesicle proteins, while the use of 16-BAC/SDS-PAGE yielded only 14 integral membrane proteins.

2.7 Evaluation

To estimate the value of the above described techniques for membrane proteomics, results have to be evaluated carefully. The number of predicted IMP has to be verified and the subcellular localization of the identified proteins confirmed.

2.7.1 Evaluation of membrane protein identification

A commonly used method to estimate the number of identified membrane proteins is to calculate the grand average hydropathy (GRAVY) index (Kyte and Doolittle, 1982) of the extracted proteins. This GRAVY value is calculated as the sum of the hydropathy values of all amino acids, divided by the number of residues in the sequence. A positive GRAVY score points at a hydrophobic protein. The score can be calculated using web-based tools such as the ProtParam tool on the Expasy server[1]. Wilkins and his colleagues determined a hydropathy cut-off point above which proteins are not longer identified on classical 2-DE maps (i.e., 0.1 for *S. cerevisiae*) (Wilkins et al., 1998).

[1] `http://www.expasy.ch/tools/protparam.html`

A better estimation is obtained by the prediction of the number of transmembrane helices (TMH) (Ephritikhine et al., 2004). Since the amino acid composition and structure of α- helices and β-strands is very typical, they can be predicted rather easily through algorithms. As already mentioned, α- helices need to be at least 15 residues long and are mainly composed of hydrophobic amino acids ,while β-strands are generally longer than 10 residues and are composed of alternating hydrophobic and polar amino acids. Even the way proteins span the membrane can be predicted since the intracellular part is generally composed of positively charged amino acids (i.e., "the positive-inside" rule). An overview of the existing databases for TMD prediction and how they function is given by Schwäcke et al. (2004) and Punta et al. (2007). However, different algorithms give different results depending on their threshold to define a region as a transmembrane region (see chapter 3). Therefore, we suggest to apply the Aramemnon[2] algorithm which uses a consensus of 18 different programs (figure 2.5).

For proteins from poorly sequenced species, only an indication of the number of TMH can be obtained since this prediction is based on the prediction of TMH of the closest homolog. In table 2.3 it is demonstrated that the predicted number of TMH of the same protein homolog differs depending on the species where the protein is derived from.

Ephritikhine et al. (2004) state that a good estimation of the power of a membrane proteomics technique is to determine the number of TMD divided by the molecular mass of the protein.

[2] `http://aramemnon.botanik.uni-koeln.de`

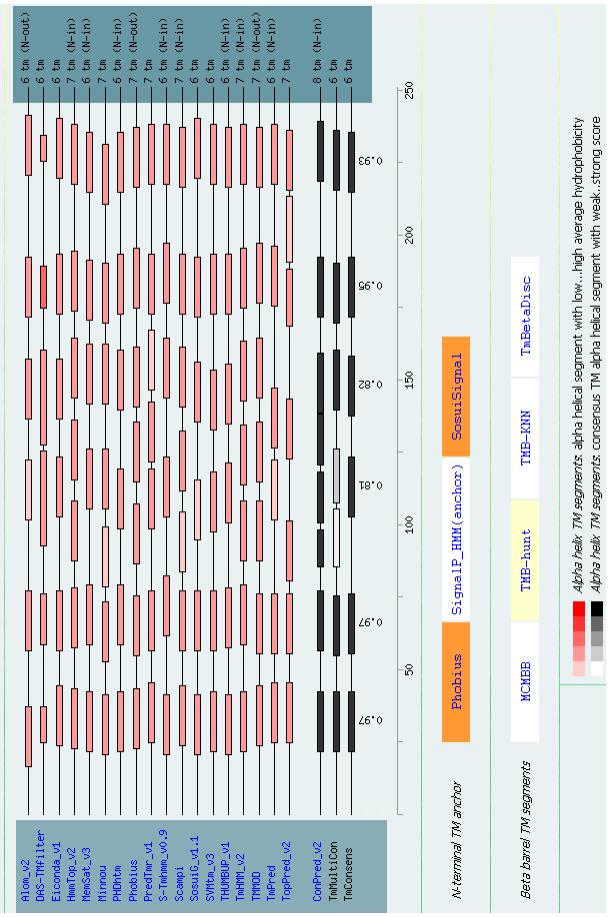

Figure 2.5: *The number of transmembrane helices present in the Arabidopsis protein At2g36830, predicted by Aramemnon, which uses a consensus (black) of 18 different programs. Bars indicate the presence of a TMH.*

Table 2.3: *Comparison of the prediction of TMH (using the prediction program Phobius* `http://phobius.cbr.su.se/`*) of the protein homologs of the Arabidopsis protein At2g36830 (see figure 2.5) from four different species. The homologs have the same amino acid number and had an E value* $< e^{-100}$ *after a blastp search (*`http://www.ncbi.nlm.nih.gov/blast/`*). In brackets the percentage of similarity with the Arabidopsis protein is shown.*

Arabidopsis			*Brassica (94%)*			*Prunus (80%)*			*Ricinus (87%)*			*Vitis (85%)*		
1	21	Non Cyto	1	21	Non Cyto	1	103	Cyto	1	19	Non Cyto	1	19	Cyto
22	44	TMH	22	44	TMH				20	37	TMH	20	37	TMH
45	55	Cyto	45	55	Cyto				38	57	Cyto	38	56	Non Cyto
56	75	TMH	56	75	TMH				58	81	TMH	57	79	TMH
76	86	Non Cyto	76	86	Non Cyto				82	86	Non Cyto	80	99	Cyto
87	108	TMH	87	108	TMH				87	109	TMH	100	124	TMH
109	114	Cyto	109	114	Cyto	104	124	TMH	110	115	Cyto			
115	135	TMH	115	135	TMH	125	143	Non Cyto	116	135	TMH	125	143	Non Cyto
136	140	Non Cyto	136	140	Non Cyto				136	140	Non Cyto			
141	162	TMH	141	162	TMH	144	163	TMH	141	162	TMH	144	164	TMH
163	173	Cyto	163	173	Cyto	164	174	Cyto	163	173	Cyto	165	170	Cyto
174	197	TMH	174	197	TMH	175	197	TMH	174	197	TMH	171	193	TMH
198	216	Non Cyto	198	216	Non Cyto	198	216	Non Cyto	198	216	Non Cyto	194	216	Non Cyto
217	237	TMH	217	237	TMH	217	237	TMH	217	237	TMH	217	239	TMH
238	251	Cyto	238	251	Cyto	238	251	Cyto	238	251	Cyto	240	251	Cyto
Tot. TMH		**7**			**7**			**4**			**7**			**6**

To estimate whether peptides from membrane domains were identified, the peptide GRAVY score and the location of its sequence within the protein sequence can be determined using the ProtParam tool.

2.7.2 Subcellular localization

When proteins from purified membrane fractions are identified, it is important to provide evidence that the protein is really associated with this membrane. A fast, cheap and not labour-intensive method is the *in silico* prediction of a protein's location. One possibility is performing a similarity search (by e.g., the BLAST algorithm[3]) thereby assuming that the same proteins share the same location in many organisms. Other prediction programs are based on the presence of known sorting signals, certain amino acid compositions, the analysis of keywords from functional annotations of proteins in publicaly available databases, evolutionary and/or structural information or a combination of the above (Guda and Subramaniam, 2005). An overview of these programs can be found in Heazlewood et al. (2005). The main disadvantage of this approach is that these programs are based on training sets of proteins with known localization. When applying these programs to uncharacterized proteins, this might lead to false predictions (Sprenger et al., 2006). Recently, a method was developed to assign multiple proteins simultaneously to a specific organelle. Localization of organelle proteins by isotope tagging (LOPIT) (Dunkley et al., 2006; Sadowski et al., 2008) uses the iTRAQ technology (see section 1.4) to determine the proteins that are located in the same organelle by using an iodixanol density gradient. First, the different organelles are distributed within this gradient by centrifugation. Subsequently, the gradient is divided in different fractions and the presence of organelle-specific markers in these fractions is determined by using antibodies against these markers. The different fractions are labeled with a different iTRAQ-label. After MS/MS analysis, the abundance of the same peptide is compared over the different fractions. Peptides that show the same pattern as the organelle specific marker are assigned to that specific organelle. Peptides from contaminating proteins will show a different pattern. Also other variants of protein correlation profiling have been described (e.g., by Andersen et al. (2003)). However, sometimes organelles are located over

[3] `http://www.ncbi.nlm.nih.gov/blast/`

different bands in the gradient which complicates the organelle targeting and makes the prediction less reliable.

Confirmation of a predicted location can be obtained using either a protein-green fluorescent protein (GFP) construct or a labeled, protein specific antibody to visualize that protein. Both methods have disadvantages; in the GFP-method the main concern is that location of the protein construct might differ from the original location because of overexpression of the protein and an abnormal protein trafficking because of the attached GFP, the use of antibodies is generally a lower throughput method (Sadowski et al., 2008).

2.8 Membrane proteomics in plants

The number of plant reports dealing with membrane proteomics is rather limited. In table A.1 in the appendix the most representative papers dealing with the characterization of the membrane proteomes are listed. When possible, the percentage of identified membrane proteins was calculated. Research that focuses on the characterization of one specific protein or protein family is not taken into account.

From this list, it is clear that research was mainly performed on the model plants *Arabidopsis* and rice. The most popular method in plants appears to be a combination of one-dimensional SDS-PAGE protein separation and a gel-free peptide separation. A logical explanation is that by using the geLC approach the high solubilizing power of SDS is used to ensure successful protein solubilization. Since separation occurs at both the protein and peptide level, resolution is improved compared to 1-D SDS-PAGE. Moreover, the most abundant proteins will be concentrated in a limited number of bands on the SDS gel which allows the identification of less abundant proteins in the other bands. Most membrane studies are performed on plasma membranes or chloroplasts. Qualitative as well as quantitative studies have been performed. Plant researchers have not explored the arsenal of alternative digestion strategies yet. Mainly trypsin is applied because of its ease of use, easy predictable cleavage sites and good ionizable peptides. Only very few reports exist on the use of alternative gel-based techniques such as 16-BAC and dSDS.

The most intensive studied protein complexes are those present in mitochondria and chloroplasts (table A.2). Mainly n-dodecyl-b-D-maltoside is used for complex solubilization because it is more efficient in solubilizing thylakoid protein complexes (see chapter 4). As can be concluded from table A.1 and A.2, the number of membrane proteomics studies on poorly sequenced plants is very limited. These studies are not straight-forward since it is recommended to use gel-based techniques (Carpentier et al., 2008b):CHeCK. Since these are all limited in resolution, performing a membrane proteomics study on non-model plants is a challenge.

In the following chapters this challenge is faced and different techniques for membrane proteomics are evaluated for the use on banana.

Part II

Experimental results

Chapter 3

Evaluation of chloroform/methanol extraction as a method to enrich samples in highly hydrophobic proteins

If you're not part of the solution, you're part of the precipitate.

Henry J. Tillman

3.1 Introduction

As explained in the introducing chapters, the best way to analyze proteins of poorly sequenced plants is by gel-based protein separation techniques combined with cross-species identification (figure 1.5) (Carpentier et al., 2008b). However, the currently available gel-based techniques for membrane proteomics do not have the excellent resolving power of classical 2-DE. In addition, the main problem in membrane proteomics is the low abundance of membrane proteins

compared to soluble proteins (see chapter 2). Enrichment in membrane proteins prior to protein separation is thus of utmost importance to increase their chance for identification (i.e. the chance their peptides have a high S/N ratio and are selected for further fragmentation in MS/MS).

Since a mixture of chloroform and methanol (C/M) is reported to specifically extract highly hydrophobic proteins with short hydrophilic domains (Ferro et al., 2000), we evaluated this method for the study of membrane proteins in banana. Chloroform/methanol extraction was presented in 1951 by Folch and coworkers as a method to extract lipids from brain tissue (Folch et al., 1951). Henriques and Park (1976) were the first to characterize proteins soluble in a C/M mixture and demonstrated that the C/M soluble fraction is enriched in proteins containing a high number of hydrophobic amino acids. The group of Joyard characterized the proteins found to be soluble in the organic phases of different ratios of chloroform to methanol (Joyard et al., 1982; Seigneurin-Berny et al., 1999). In the following years, the method was applied to study hydrophobic proteins of purified chloroplast envelope (Ferro et al., 2002, 2003) and thylakoid (Friso et al., 2004) membranes, and of mitochondrial (Brugière et al., 2004), tonoplast (Schmidt et al., 2007) and plasma membranes (Marmagne et al., 2004). In this chapter chloroform/methanol extraction is evaluated as a method to analyze membrane proteins from total cellular lysates of a poorly sequenced plant. We prefer total cellular lysates as starting material to study membrane proteins from the different organelles simultaneously. Moreover, meristems are scarce tissue which makes isolation of sufficient amounts of individual organelles troublesome. The method is applied on leaves of a sequenced (*Arabidopsis*) and a poorly sequenced (banana) plant to demonstrate the difficulties associated with proteomics studies on poorly sequenced plants and to make a comparison with results from literature possible. For protein separation, the resolution of one- and two-dimensional SDS-PAGE is compared. Subsequently, proteins from banana shoot apical meristems (*Musa* spp.) are extracted in a C/M mixture to characterize the proteome of meristem membranes. All together, the chapter provides an overview of the merits and disadvantages of C/M extraction and estimates its value for membrane proteomics studies on poorly sequenced plants.

3.2 Materials and methods

3.2.1 Chemicals and materials

Unless otherwise indicated, all chemicals used in this and the following chapters were from Sigma (St. Louis, MO) or GE Healthcare (Uppsala, Sweden).

3.2.2 Plant material

Arabidopsis thaliana var. *Columbia* plants, which were kindly provided by F. Rolland (K.U.Leuven, Belgium), were grown using a daily cycle of 12 h light (75 µmol m^{-2} s^{-1}) at 22 ℃ and 12 h darkness at 18 ℃ and 50% relative humidity. After 2 months, leaves were harvested and immediately frozen in liquid nitrogen. Banana plantlets were obtained from the Bioversity International *Musa* collection at K.U.Leuven, Belgium. Banana plants were grown in the greenhouse with 27 and 20 ℃ of respective day and night temperatures, a 12 h photoperiod and 80% relative humidity. Leaves were collected from 1 year old plants. Multiple shoot meristem cultures were initiated as described by Strosse et al. (2006) and subsequently maintained on a standard control medium containing 0.09M sucrose (i.e. Murashige and Skoog medium supplemented with benzylaminopurine). All cultures were kept in the dark at 25 ℃. After one month of culture, meristems were grinded in liquid nitrogen.

3.2.3 Protein extraction

After grinding in liquid nitrogen, 200-400 mg material (fresh weight) was transferred to 1 mL ice cold extraction buffer, containing 100 mM Tris-HCl (pH 8.3), 5mM EDTA.Na_2, 100 mM KCl (Acros, Geel, Belgium), 1% DTT and complete protease inhibitor cocktail (Roche, Mannheim, Germany). Samples (100 µL) were added to an ice-cold chloroform (Chem-Lab, Zedelgem, Belgium)/methanol (VWR, Amsterdam, Netherlands) mixture in a ratio 1/9 as described by Seigneurin-Berny et al. (1999) and carefully mixed. Different C/M ratios were tested. Samples were incubated on ice for 30 min and centrifuged at 16, 000 *g* (4 ℃) for 1 h. Pellets were washed overnight in ice-cold acetone containing 0.2% DTT. Organic phases were collected and proteins that were soluble in these phases were precipitated overnight at -20 ℃ by addition of 1 mL cold di-ethylether. After centrifugation at 4 ℃ (16, 000 *g* for 1 h), pellets

were solubilized in 100 mM Tris-HCl (pH 6.8), 4% SDS (Bio-Rad laboratories, Hercules, CA, USA). Total protein extract was retrieved after trichloroacetic acid (TCA) (VWR) precipitation. One hundred µL of sample was added to 1.9 mL 10 % TCA in ice cold acetone with 0.2% DTT and precipitated overnight. After 1 h centrifugation at 16, 000 *g* (4 ℃), pellets were washed with ice-cold acetone containing 0.2% DTT. Pellets were dissolved in the same SDS buffer as the C/M soluble and insoluble proteins. The protein concentration was measured using a micro-Bradford membrane protein assay as described by Zuo and Lundahl (2000) and samples were stored at -80 ℃.

3.2.4 Protein separation

After thawing, loading buffer was added to reach a final concentration of 3% SDS, 75 mM Tris-HCl (pH 6.8), 15% glycerol, 3.5 M urea, 1% DTT and 0.05% bromophenol blue. Samples were heated at 37 ℃ for 30 min and briefly centrifuged. Equal amounts (40 µg) of proteins were loaded and proteins were separated via SDS-PAGE. For dSDS separation of banana leaf proteins, 90 µg of sample was loaded. For all separations the Laemmli protocol (Laemmli, 1970) was used.

3.2.4.1 1-D separation

For one dimensional separation, a 10-15.5 % hyperbolic gradient gel (18 × 24 cm, 1.5 mm), generated by a 2DE optimizer (NextGen Sciences, Alconbury, UK) was used. The stacking gel consisted of 4% acrylamide (Bio-Rad laboratories, Hercules, CA, USA). Gels were run overnight at 2W/gel at 12 ℃.

3.2.4.2 2-D separation

For dSDS-PAGE, gels were poured manually. The separating gel of the first dimension (18 × 24 cm, 1 mm) consisted of 10 % acrylamide, while the second dimension separating gel (26 × 20 cm, 1.5 mm) contained 15 % acrylamide. Both stacking gels contained 4% acrylamide. The dSDS protocol was performed as described by Rais et al. (2004) with slight modifications. Urea was omitted from the gel since the identification of highly hydrophobic proteins was desired (Rais et al., 2004). After a first dimension separation, lanes were excised, swollen for 45 min in buffer (100 mM Tris, 0.2% DTT, pH 2.0) and placed

on top of the second dimension gels. Gaps between the excised gel lane and the spacer were filled with agarose sealing solution containing 0.5% agarose (Gentaur, Kampenhout, Belgium), 0.002% bromophenol blue and 1 × Laemmli buffer. The first dimension was run overnight at 2W/gel at 12 ℃. Second dimension gels were run at 20 ℃. For entering and protein migration through the stacking gel 2W/gel was applied. After 1.5 h, the power was increased to 16.7 W/gel. All gels were stained with G-250 Colloidal Coomassie (Serva, Heidelberg, Germany)(Neuhoff et al., 1988).

3.2.5 Image analyses

Gel images were captured with labscan 5 software (GE Healthcare) and analysis of 1-D and 2-D images was performed using Quantity One (Bio-Rad) and Image Master 2-D platinum (GE healthcare) software, respectively.

3.2.6 Protein identification

After Coomassie blue staining and image analysis, spots were manually picked. In-gel digestion with trypsin and analysis of the tryptic peptides by MALDI TOF-TOF (4800 from Applied Biosystems, Foster City, CA, USA) was performed at the C̈entre de Recherche Public Gabriel Lippmann" in Luxembourg. For digestion, the Ettan dalt spot handling workstation (GE Healthcare) was used. After reduction and alkylation, gel pieces were washed and desalted first in 50 mM AmmBic/50% v/v methanol and subsequently in 75% v/v acetonitrile (ACN). After adding 8 µL of a Trypsin Gold solution (5 ng/mL) in 20 mM AmmBic (Promega, Madison, WI, USA) to the dried gel plugs, samples were incubated at 37 ℃ for six hours. After extraction and drying, the resulting peptides were dissolved in 3 µL of a 50% ACN solution containing 0.1% trifluoroacetic acid (TFA) and 0.7 µL of each well was spotted on disposable MALDI-TOF target plates. Spotted peptides were mixed with 0.7 µL of α-cyano-4-hydroxycinnamic acid (7 mg/L, 50% ACN/0.1% TFA) and allowed to air-dry. Mass spectrometrical analyses (MS and MS/MS) were carried out using the Applied Biosystems 4800 Proteomics Analyzer (Applied Biosystems) in positive electron mode, externally calibrated using the peptide mass calibration kit, according to the manufactures instructions (Applied Biosystems). An in-house Mascot platform was used for searching against the NCBI *Viridiplantae* and the *Musa* EST database. The searching parameters allowed 2 missed

cleavages, a tolerance of 0.50 Da on MS/MS fragments and 100 ppm on precursor mass, as well as carbamidomethylation on cysteine as a fixed modification. Double oxidation and kynurenine formation of tryptophan and oxidation of methionine were allowed as variable modifications. The probability score (Mowse score (Perkins et al., 1999)) calculated by the software was used as a criterion for accurate identification. Estimation of false positive rates was made by searching a decoy database with the same search criteria. This decoy database was composed of the proteins present in the NCBI *Viridiplantae* database and from which the amino acid composition was randomly scrambled. More information on protein and ion scores and peptide sequences can be found in the supplementary material of this chapter.

3.2.7 Prediction methods

Identifications obtained by the Mascot searches were searched in batch against the Swiss-Prot database using the blastcl3 tool which interacts directly with the NCBI Blast server[1]. The ProtParam tool of the ExPASy server[2] was used to calculate the grand average of hydropathicity (GRAVY) score (Kyte and Doolittle, 1982) and other parameters such as the theoretical pI and M_r. The number of transmembrane domains was calculated by the TMHMM Server v 2.0[3], Phobius[4], HMMTOP[5] (Tusnady and Simon, 2001) and the Aramemnon[6] website. Sequence alignments were performed using the ClustalW2 tool of the European Bioinformatics Institute[7]. Protein location was predicted using the Target P server[8] and functional domains were predicted by Pfam[9]. Information on *Arabidopsis* proteins was obtained from the Aramemnon and TAIR[10] website. Results were evaluated and updated according to the information found in the plant proteome database (PPDB)[11].

[1] http://www.ncbi.nlm.nih.gov/staff/tao/URLAPI/netblast.html
[2] http://ca.expasy.org/
[3] http://protfun.net/services/TMHMM/
[4] http://phobius.cbr.su.se/
[5] http://www.enzim.hu/hmmtop/
[6] http://aramemnon.botanik.uni-koeln.de/
[7] http://www.ebi.ac.uk/Information/
[8] http://www.cbs.dtu.dk/services/TargetP/
[9] http://pfam.sanger.ac.uk/
[10] http://www.arabidopsis.org/
[11] http://ppdb.tc.cornell.edu/

3.3 Results and discussion

3.3.1 Chloroform/methanol extraction on whole leaf lysates

As stated in the introduction, chloroform/methanol extraction is generally performed on isolated organelles or membranes. Since we want to have an overview on the complete membrane proteome of banana tissue, we preferred to apply the method on total cellular lysates. Moreover, meristems are scarce tissue and purification of organelles requires large amounts of plant material. Chloroform/methanol extraction was first applied on leaves of a sequenced (*Arabidopsis*) and a poorly sequenced (banana) plant. By comparing both extractions, we wish to demonstrate the difficulties associated with studies on poorly sequenced plants. Due to the absence of proliferating meristem clumps from *Arabidopsis*, such comparison can not be performed at the level of meristems. Moreover, performing the extraction on leaf material also allows making a comparison between the results obtained from total cellular lysates and those obtained from published chloroplast research.

Since C/M extraction was not yet evaluated for use on whole cell lysates, the ratio of chloroform to methanol (ranging from 0/9 to 8/1) yielding the highest amount of proteins was determined for *Arabidopsis* as well as for banana, as recommended by Rolland and coworkers (Rolland et al., 2006). As illustrated in figure 3.1, only in the C/M ratios of 3/6 to 6/3, a considerable amount of proteins remained soluble in the organic phase, irrespective of the plant species. Since the 5/4 ratio resulted in the highest number of bands and the highest total peak intensity, this ratio was chosen for further analyses for both *Arabidopsis* and banana. As predicted by Bligh and Dyer (Bligh and Dyer, 1959), only one organic phase and a white pellet containing the insoluble proteins was obtained using this ratio. Since also lipids and pigments were extracted in the organic phase, they were further eliminated through protein precipitation with di-ethylether.

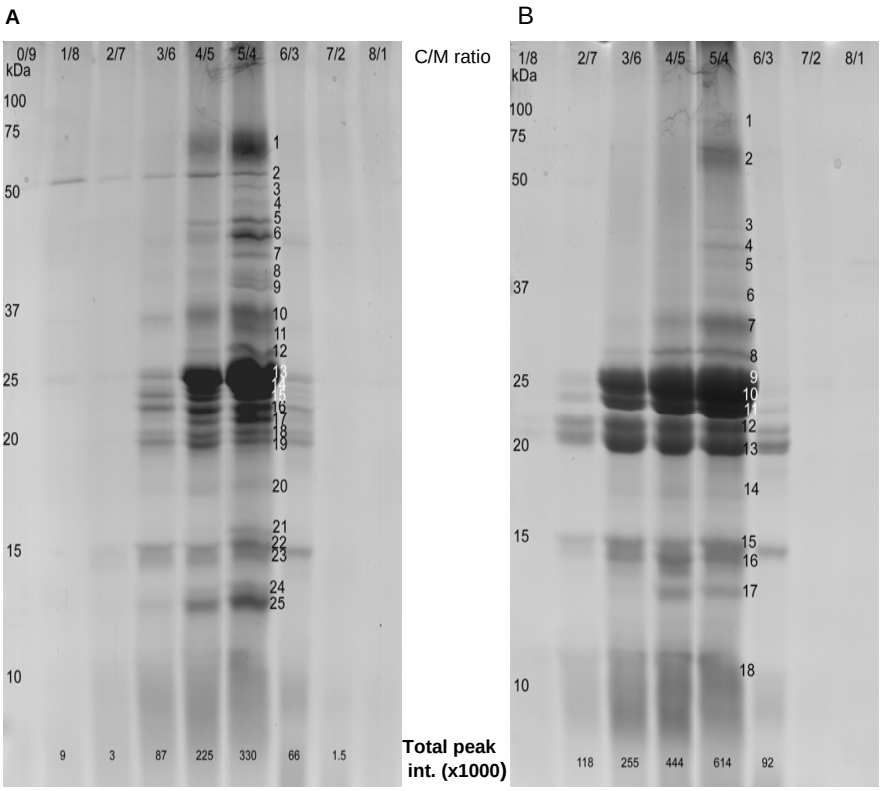

Figure 3.1: *Proteins from Arabidopsis (A) or banana (B) leaves, extracted in organic phases of different chloroform to methanol ratios separated on a 10-15.5% (hyperbolic acrylamide gradient) SDS gel and stained with Coomassie Brilliant Blue. Molecular masses (kDa) of a protein standard are indicated on the left, C/M ratios are shown on top. At the bottom the total peak intensity (x1000) as calculated by the Quantity One software (Bio-Rad) is given. The numbers are indicative for the bands which were excised for protein identification (tables 3.2 on page 76 and 3.3 on page 81).*

3.3.1.1 *Arabidopsis*: proteomics on a sequenced plant

A. Increase in abundance of membrane proteins by C/M extraction
One of the major problems to identify membrane proteins in whole cell lysates is their low abundance compared to water soluble proteins. It was therefore important to evaluate whether C/M extraction indeed resulted in an extract that is enriched in membrane proteins and depleted in highly abundant water soluble proteins. The total protein extract from *Arabidopsis* leaves was com-

pared with the proteins that are soluble and insoluble in C/M (figure 3.2). Experiments were performed on *Arabidopsis* because the use of a sequenced plant ensures a higher identification rate.

Some representative proteins were identified as proof of principle (table 3.1).

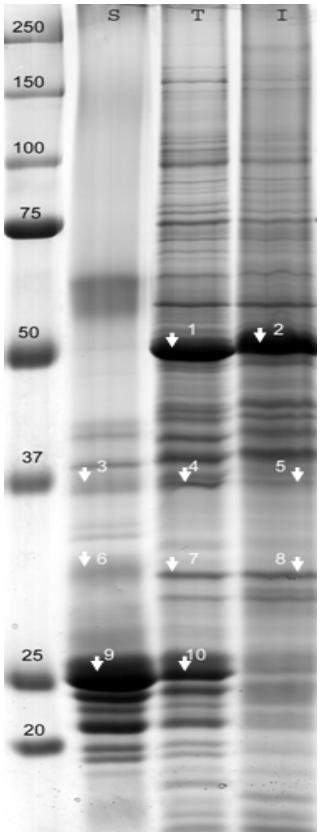

Figure 3.2: *Proteins soluble (S) and insoluble (I) in a 5/4 C/M mixture compared to the total protein content (T) of Arabidopsis leaves on a uniform 10% acrylamide gel. 40 µg of proteins was loaded. Gel was Coomassie Brilliant blue stained. Molecular masses of the protein standard (kDa) are indicated on the left. Some representative bands (numbered) were excised for protein identification (table 3.1).*

The most abundant protein in the total leaf (T) as well as in the C/M-insoluble (I) extract was identified as the large chain of Rubisco (figure 3.2, band numbers 1 and 2). It was almost completely absent from the C/M-soluble (S) fraction. The most heavily stained bands of the C/M-soluble fraction contained proteins associated with light harvesting complex II such as chlorophyll a/b binding proteins (band number 9). Those membrane proteins were clearly depleted from the insoluble fraction but could be identified in the total protein fraction (band number 10). Also other membrane proteins, such as the photosystem II 44 kDa protein could be identified in both the C/M-soluble and total cell lysate fractions (band numbers 3 and 4 resp.). Less abundant integral membrane proteins such as photosystem II Qb protein (band number 6) could only be detected in the C/M-soluble fraction. This was indicative for an enrichment in integral membrane proteins in the C/M-soluble fraction.

Table 3.1: *Proteins identified in the bands depicted on figure 3.2. Accession number (Swiss-Prot), protein name, protein score (Mascot), the number of non-redundant peptides identified per protein (NR), the band where the protein was identified, GRAVY score and number of transmembrane helices (TMH; determined by TMHMM v2.0) are shown. S = proteins soluble in a 5/4 C/M mixture; T = total protein extract after TCA precipitation; I = proteins insoluble in a 5/4 C/M mixture. The FDR was estimated 0 at the MS/MS level for all identifications reported in this chapter.*

Acc.	**Name**	**Score**	**NR**	**Band number**			**GRAVY**	**TMH**
				S	**T**	**I**		
O03042	Rubisco large chain	404	5	/	1	2	-0.272	0
P56778	Photosystem II 44 kDa protein	256	4	3	4	/	0.252	6
Q944G9	Probable fructose-bisphosphate aldolase	243	5	/	4	5	-0.167	0
Q9MAWO	Photosystem II Qb protein	223	4	6	/	/	-0.062	8
P23321	33 kDa oxygen-evolving protein	334	5	/	7	8	-0.327	0
P04777	Chlorophyll a/b binding protein	450	7	9	10	/	-0.056	2

B. Resolution: one dimensional gradient SDS versus dSDS-PAGE
The main constraint for protein identification in poorly sequenced plants is the lack of genomic information as explained in chapter 1. Hence, a good protein separation is indispensable. For that reason, we evaluated whether the resolution of a one dimensional separation (figure 3.1A (5/4 lane)) or two dimensional separation (figure 3.3) was sufficient to ensure protein identification of the C/M extracted proteins.

The acrylamide concentration of the first dimension gel was adapted to make optimal use of the total length of the gel. From figure 3.2 it is clear that mainly low molecular weight proteins were extracted in a mixture of chloroform and methanol. Therefore a 10-15.5% hyperbolic acrylamide gradient was utilized. Double SDS (see chapter 2) was chosen as two dimensional technique since it is considered to be superior to separate and visualize highly hydrophobic proteins in comparison with the use of for example benzyldimethyl-n-hexadecylammonium chloride (16-BAC)-gels (Burré et al., 2006). Twenty five bands were picked from the first dimension gel (figure 3.1A), 23 gave rise to identifications and in 48 % (11 out of 23) of the bands more than one protein was identified (see table 3.2). From the dSDS gel, 29 spots were picked, 27 resulted in identifications and only in 19 % of the spots (5 out of 27) two or more proteins were identified. This is a good indication that implementing a second dimension improved resolution and will thus simplify the final tryptic digest. Moreover, dSDS allowed the study of protein isoforms like the mitochondrial and peroxisomal form of NAD-dependent malate dehydrogenase (figure 3.3). They were detected in two different spots on the dSDS gel (spots 48 and 49 respectively) while on the 1-D gel they were located in one band (band 8).

However, the application of dSDS also has its disadvantages: the resolution remains limited (Rabilloud et al., 2008) and the introduction of an extra dimension is inevitably associated with loss of proteins. Due to this protein loss and the fact that protein identification depends on protein abundance and peptide intensities, protein identifications after one- or two- dimensional SDS-PAGE complemented each other.

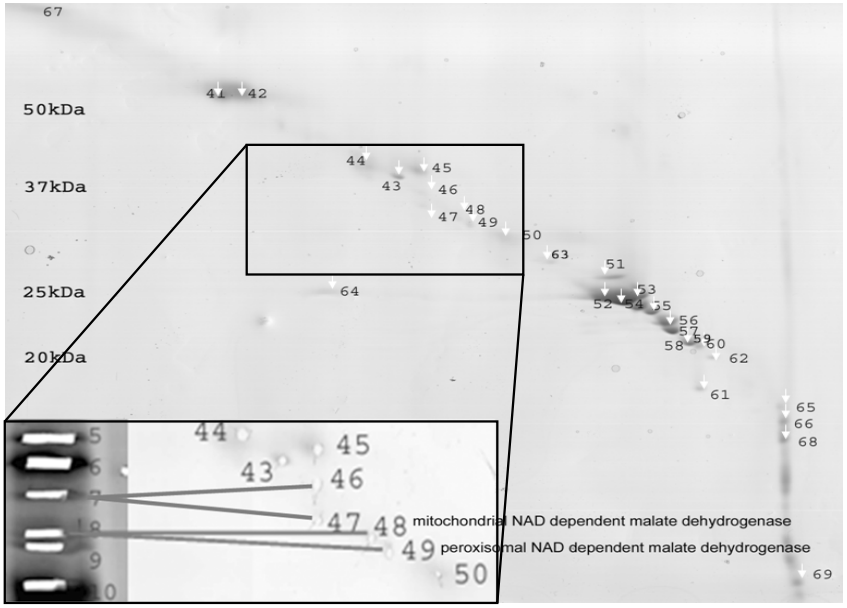

Figure 3.3: *Double SDS gel (CBB stained) of proteins (40 µg) from Arabidopsis leaves soluble in 5/4 C/M. Numbers indicate the number of the spot, corresponding to one or more identified proteins. A molecular mass standard is indicated on the left. The magnification (down left) corner shows that proteins that are detected in one band on the 1-D gradient gel (figure 3.1A, band 8) are dispersed in two spots on the 2-D gel.*

In total, the extraction of *Arabidopsis* leaf proteins in a 5/4 C/M mixture and 1-D and dSDS separation yielded 36 non-redundant protein identifications (table 3.2). This number is of the same order of magnitude as the 37 proteins identified by Ferro and coworkers in a study of *Arabidopsis* chloroplasts (Ferro et al., 2003) and the 31 proteins reported by Brugière et al. (2004) in a study of the mitochondrial proteome. This underlines that the C/M method is very selective.

Table 3.2: *Proteins identified in the 5/4 C/M-soluble fraction of Arabidopsis leaves. Shown are: the Arabidopsis gene identifier (Agi), protein description, protein score (Mascot), the NR identified per protein, the predicted relative molecular mass M_r, iso-electric point pI, the number of TMH (determined by Aramemnon), the location as present in the PPDB (C= chloroplast, M= mitochondrion, P= peroxisome,O= other), the band (1-D)/spot (dSDS) where the protein was identified and whether the protein was also identified on the map of Giavalisco et al. (2005) (2-DE).*

Agi	Protein	Score	NR	M_r	pI	TMH	GRAVY	Location	1-D	dSDS	2-DE
AT1G53240.1	Malate dehydrogenase	352	5	35.81	8.54	0	0.165	M	8	48	X
AT2G44350.1	Citrate synthase	199	4	52.66	6.41	0	-0.204	M	4		X
AT2G13360.1	Ala-glyoxylate aminotransferase	510	4	44.21	7.69	0	0.023	P	5	44	
AT3G14415.1	Glycolate oxidase	510	6	40.31	8.99	0	-0.031	P	6	43	
AT5G09660.1	Malate dehydrogenasel	650	6	37.37	8.14	1	0.096	P	8,9	49	X
AT2G37660.1	3-β-hydroxy-δ5-steroid dehydrogenase	173	2	34.88	8.37	0	-0.18	C	11		X
AT2G25080.1	Glutathione peroxidase	137	1	26.02	9.42	0	-0.174	C	20		
AT1G67090.1	Rubisco small subunit	501	3	20.22	7.59	0	-0.267	C	25		X
AT4G38970.1	Fructose-bisphosphate aldolase	472	5	42.99	6.79	0	-0.167	C	7	46,47	X
AT3G11630.1	2-Cys Peroxiredoxin A	210	2	29.09	6.91	0	-0.112	C	17	57	X
Plastid gene	Rubisco large chain	376	5	52.96	5.88	0	-0.253	C	2		X
ATCG00340.1	PsaB - subunit Ib	202	4	82.48	6.89	11	0.12	C	1	41,42	
AT5G01530.1	LHCII-4.1-CP29	311	3	31.14	5.76	2	-0.021	C	12		
AT3G08940.1	LHCII-4.2 - CP29	257	4	25	7.71	2	0.024	C	12	51	
AT1G29930.1	LHCII-1.3	279	2	28.24	5.47	3	0.021	C	13	52,64	
AT1G29920.1	LHCII-1.2	218	2	28.23	5.29	3	0.022	C	13	63	
AT3G27690.1	LHCII-2.4	136	2	28.8	5.62	2	-0.121	C	13	64	
AT3G47470.1	LHCI-4	488	3	27.73	6.22	0	-0.156	C	18	58	
AT3G54890.1	LHCI-1-1	439	1	26	6.21	2	-0.074	C	19	60,61	
ATCG00130.1	CFO-I - atpF	185	2	21.06	7.85	1	-0.392	C	21	65	
ATCG00270.1	PsbD D2	375	3	39.55	5.46	6	0.372	C	1,3,10,11	67	
AT1G61520.1	LHCI-3 (CAB4)	439	4	29.18	8.61	1	-0.014	C	15,21	55	X
AT1G15820.1	LHCII-6 (CP24)	469	4	27.52	6.75	2	0.033	C	16,17	56,57	
AT4G03280.1	PetC - Rieske Fe-S protein	332	4	24.37	8.8	1	-0.087	C	20		
AT4G21280.1	PsbQ-1	194	2	23.8	9.64	1	-0.32	C	22		X
AT4G05180.1	PsbQ-2	662	5	24.64	9.72	0	-0.24	C	22,23	68	X

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Agi	Protein	Score	NR	M_r	pI	TMH	GRAVY	Location	1-D	dSDS	2-DE
AT5G02240.1	NAD dependent epimerase/dehydratase	326	5	27.1	6.2	0	-0.272	C	11	59	
AT5G54270.1	LHCII-3	165	4	28.71	4.96	2	0.034	C		54	
AT4G10340.1	LHCII-5 (CP26)	657	6	30.16	6	2	-0.025	C		53	
ATCG00350.1	PsaA - subunit Ia	355	6	83.23	6.6	11	0.252	C		42	
ATCG00280.1	PsbC (CP43)	355	6	51.87	6.71	6	0.252	C		44,45	
AT3G61470.1	LHCI-2.1	209	2	27.76	6.91	0	-0.001	C		56	
AT1G31330.1	PsaF- subunit III	176	2	24.17	9.58	2	-0.011	C		69	
AT4G12800.1	PsaL - subunit XI (also named V)	234	4	23.05	9.85	2	0.31	C		66	
AT1G23310.2	Ala 2-oxoglutarate amino transferase	254	4	48.55	8.58	0	-0.159	O	3		
AT4G23600.1	Nicotianamine aminotransferase	220	3	47.04	5.9	0	-0.141	O	4		

As already observed by Henriques and Park in 1976 (Henriques and Park, 1976) and Brugière and coworkers in 2004 (Brugière et al., 2004) and as stated above only proteins with a low M_r were present in the C/M-soluble phase. High M_r proteins probably precipitate during C/M extraction. Schröder and coworkers proposed the addition of a halogenic acid and an extra phase separation to recover these high M_r membrane proteins (Schröder and Hasilik, 2006). However, this extra phase separation was associated with additional significant protein losses which prevented mass spectrometric identification (Schröder et al., 2007). Moreover, we observed that after the addition of the acid also many soluble proteins were re-extracted. Therefore, we do not recommend implementing this step.

C. Chloroform/methanol extraction versus classical 2-DE The main motivation to perform C/M extraction combined with gel electrophoresis was to complement classical 2-DE studies and to find an approach to study membrane proteins in poorly sequenced plants. For a better insight in the hydrophobic character of the identified proteins, the average GRAVY score was calculated and turned out to be slightly negative (-0.047). The negative average score can be explained by the presence of some highly hydrophilic contaminants such as the Rubisco large and small chains (resp -0.253 and -0.267). Although comparison of a total leaf extract and the C/M-soluble fraction clearly demonstrated a severe depletion of these highly abundant proteins in the latter fraction (figure 3.2), the presence of these proteins seems to be inevitable (Ferro et al., 2003). A more convenient approach to evaluate whether membrane proteins were indeed identified relies on the calculation of the number of transmembrane helices (TMH). Different prediction programs are available of which TMHMMv2.0 is reported to be the best performing (Möller et al., 2001). The TMHMMv2.0 server predicted that only 22% of the C/M-soluble proteins were transmembrane proteins. The plant specific membrane database, Aramemnon, uses a consensus of 17 prediction programs and predicted that 58% of the obtained proteins were integral membrane proteins (Schwäcke et al., 2003). Using the HMMTOP server (Tusnady and Simon, 2001), the number of transmembrane proteins was much higher and reached 72%. These differences clearly demonstrate that setting the threshold of the prediction too strict might result in false negative results while setting it too broad might result in false positives. Therefore, Aramemnon was preferred. However, *in silico* predictions should be validated by localization studies (see chapter 2, subsection 2.7.2) which will be instrumental to improve the algorithms and make them more plant specific.

We compared our results with a published 2-DE map of *Arabidopsis* leaf proteins. Giavalisco and coworkers published such a map in 2005 (Giavalisco et al., 2005). Although the authors took special precautions to increase the number of membrane proteins on the 2-DE gels, proteins containing more than one transmembrane helix were not identified. Seventy percent of our proteins extracted with C/M could not be found on this 2-DE map which confirms C/M extraction complements classical 2-DE.

To estimate the reproducibility of the method and the value of performing chloroform/methanol extraction on whole cell lysates, a comparison was made between the results shown in table 3.2 and previously published results on C/M extraction. First, the location of the identified proteins was determined. Protein location was predicted by TargetP. Since the server is estimated to give 10% false predictions (Peltier et al., 2004), the location prediction was evaluated by using the information in the PPDB. Most of the identified proteins were associated with the chloroplast thylakoid membrane (figure 3.4A) and were involved in photosynthesis (78%), indicating that C/M extraction, like most proteomics approaches, has a bias toward abundant proteins. A detailed study of the location and function of the obtained proteins was not performed as protein functions are discussed in published papers such as Ferro et al. (2003) and Friso et al. (2004).

3.3.1.2 Banana: proteomics on a poorly sequenced plant

To demonstrate the differences between proteomics on a sequenced and poorly sequenced plant, the same protocol was performed on banana leaves. In total, 20 non-redundant proteins were identified in the C/M banana leaf extract using both the one- and two dimensional approach (table 3.3). This corresponds to an average identification rate of 61%, which was comparable to the identification rate in our 2-DE analyses of banana (Carpentier et al., 2008a) but which was significantly lower than the 92.5% obtained with *Arabidopsis* .

Using cross-species identification and the Aramemnon database, 70% of the identified proteins were predicted to be transmembrane proteins. However, as stated above, care should be taken to consider a protein as being a real transmembrane protein, especially when working with poorly sequenced organisms. Only the closest homolog is identified based on the similarity with identified

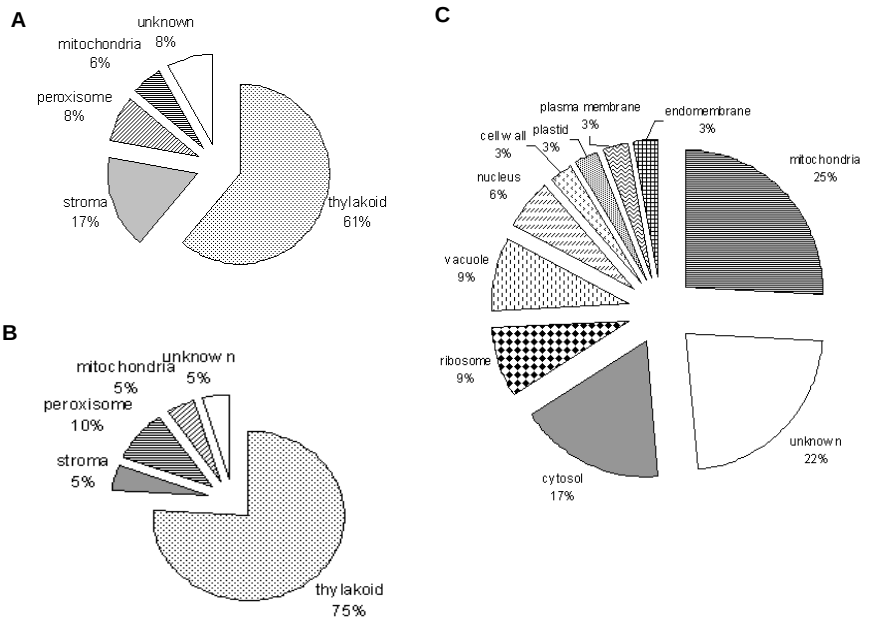

Figure 3.4: *Location of Arabidopsis leaf (A) or banana leaf (B) and meristem (C) proteins extracted in a 5/4 C/M mixture. Information was retrieved from the Target P server (*`http://www.cbs.dtu.dk/services/TargetP/`*) and the plant proteome database (*`http://ppdb.tc.cornell.edu`*).*

peptides. Since trypsin was used for protein digestion, these peptides are generally located outside transmembrane regions. It is thus difficult to exactly predict the number of TMH from proteins from poorly sequenced species.

Like in *Arabidopsis*, most proteins were associated with chloroplast thylakoids and participated in the photosynthesis process. The majority of the identified proteins (table 3.3) could not be located on our previously generated 2-DE map of banana leaf proteins (Carpentier et al., 2008a). This map, like most 2-DE maps, did not reveal membrane proteins with more than one transmembrane domain. Only the putative serine-glyoxylate aminotransferase, peroxisomal malate dehydrogenase and Rubisco small subunit were detected using both classical 2-DE and C/M extraction combined with gel electrophoresis. These results confirm the complementarity between C/M extraction and classical 2-DE.

Table 3.3: *Proteins identified after C/M extraction of a whole banana leaf lysate combined with one dimensional gradient (1-D) or dSDS (2-D) PAGE. The Swiss-Prot or Musa EST accession number, the closest protein homolog, protein score (Mascot), the NR per protein, the predicted physicochemical properties; relative molecular masses (M_r, × 1000), pI, the number of transmembrane helices (TMH, determined by Aramemnon), GRAVY scores and the location (C= chloroplast, M= mitochondrion, P= peroxisome and O= other) are shown. The number of the bands on the gradient gel (1-D, figure 1B) or the spots on the dSDS gel (2-D, figure A.1 in the appendix) of the identified proteins are indicated as well as whether the proteins were also identified on a classical 2-DE map of banana leaf proteins (2-DE) (Carpentier et al., 2008a).*

Accession	Closest homolog	Score	NR	M_r	p*I*	TMH	GRAVY	Location	1-D	2-D	2-DE
Q40433	Photosystem I psaH protein [*Nicotiana sylvestris*]	78	1	15.3	9.95	0	-0.134	C		44	
O24045	Rubisco small subunit [*Musa acuminata*]	100	42	20.5	8.78	0	-0.242	C	17	42	x
Q9SUI4	PS I reaction center subunit XI [*Nicotiana attenuata*]	108	1	23.1	9.85	0	0.31	C	15		
Q41039	Lhca4 [*Pinus sylvestris*]	245	3	26.8	7.12	0	-0.093	C		21	
Q94JA2	Malate dehydrogenase [*Oryza sativa*]	218	3	35.5	8.74	0	0.076	M		37	
O49124	Putative serine-glyoxylate aminotransferase [*Fritillaria agrestis*]	187	3	44.1	7.63	0	-0.012	O	3	47	x
Q6V8T3	Chlorophyll a/b-binding protein type I [*Malus x domestica*]	230	3	15.6	5.05	1	0.116	C		22	
MUSF352TF	Rieske FeS protein precursor	138	1	23.9	8.55	1	-0.08	C	14		
O64450	Lhcb1*9 [*Nicotiana sylvestris*]	146	2	28.3	5.48	1	0.016	C		29	
P93260	Glycolate oxidase [*Mesembryanthemum crystallinum*]	337	4	31.3	9.16	1	0.011	P	4	36	
Q0ILQ0	Malate dehydrogenase [*Oryza sativa*]	135	34	37.4	8.09	1	0.181	P	6	34	x
Q67HN4	Cytochrome b-559 alpha subunit [*Cartonema philydroides*]	225	4	8.6	4.75	2	0.193	C		45	

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Accession	Closest homolog	Score	NR	M_r	p*I*	TMH	GRAVY	Location	1-D	2-D	2-DE
MUSO477TF	PS I reaction center subunit V [*Nicotiana attenuata*]	244	3	13.2	10.47	2	0.192	C	18		
P15192	Chlorophyll a-b binding protein type 2 member 2 [*Pinus sylvestris*]	172	2	16.1	4.79	2	0.156	C		28	
P36494	Chlorophyll a-b binding protein CP24 [*Solanum lycopersicum*]	94	2	27.8	6.15	2	0.085	C		24	
Q6ZF30	Putative chlorophyll a/b binding protein of LHCII type III [*Oryza sativa*]	282	2	28.8	5.82	2	0.025	C	11	26	
1908421A	Light-harvesting complex IIa protein [*Hordeum vulgare*]	178	2	31.3	5.33	2	-0.149	C		30	
P05643	Cytochrome b6/f complex subunit IV [*Zea mays*]	124	1	17.5	6.56	3	0.551	C	15		
Q8HTU2	Photosystem II D2 protein [*Columnea sp. Lindqvist and Albert 30*]	261	3	83.2	6.66	9	0.372 6, 7	C	1,2,5,		
Q7YJY8	Photosystem II D1 protein [*Calycanthus floridus*]	225	4	82.5	6.89	11	0.12	C		33	

The identification of the Lhcb1*9 isoform demonstrates the protein inference problem (Nesvizhskii and Aebersold, 2005) (see subsection 1.3.2) and the need of an increased availability of DNA sequences of more plant species. The plant kingdom is characterized by the existence of many multigene families which give rise to protein isoforms. In case the protein isoform of a poorly sequenced plant slightly differs from that of a sequenced plant, there is a chance that peptides with the highest S/N are derived from a region that differs in amino acid sequence. Indeed, the peptides that led to the identification of Lhcb1*9 differ from the homologous peptides of the five *Arabidopsis* Lhcb1 isoforms (figure 3.5).

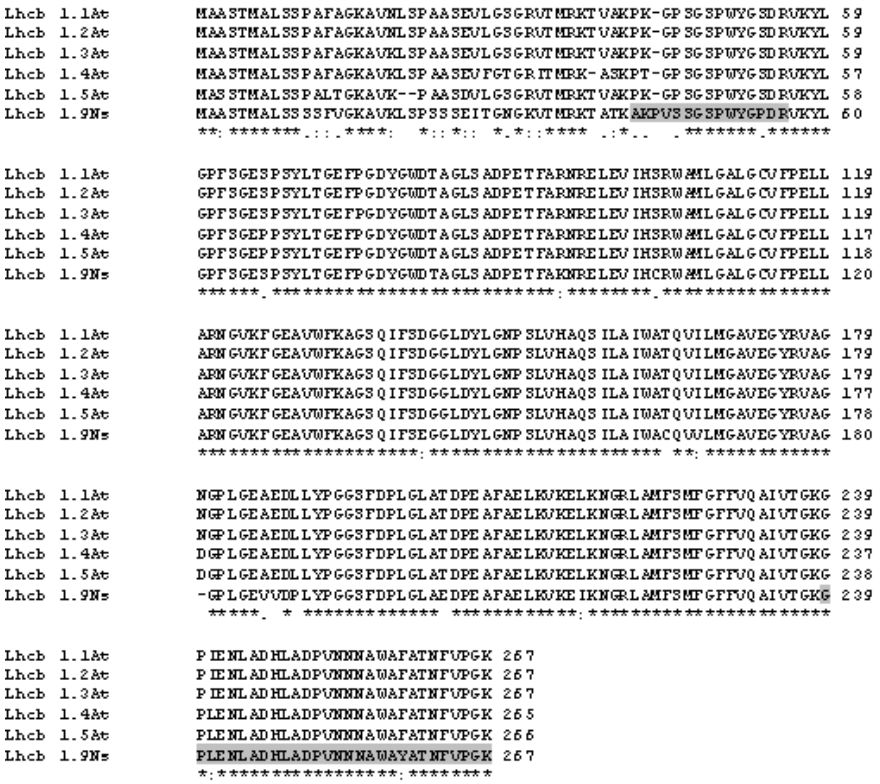


Figure 3.5: *Clustal W alignment of the 5 different Lhcb1 isoforms of Arabidopsis (At) and the Lhcb1*9 isoform of Nicotiana sylvestris (Ns). The peptides that were decisive for MS/MS based identification are indicated and are located in a mutated region of the protein.*

3.3.2 Chloroform/methanol extraction on whole meristem lysates

Although less protein identifications were retrieved from banana leaves compared to *Arabidopsis* leaves because of the poor sequencing status of banana, C/M extraction in combination with gel electrophoresis proved to complement classical 2-DE studies, also in banana. Since we aimed at broadening the known meristem proteome, we applied the method on banana meristems. It would also allow to determine whether C/M preferentially extracts proteins associated with chloroplasts.

First, the optimal C/M ratio was determined as described above for leaves (data not shown). Again, the 5/4 C/M ratio was selected. Extracted proteins were separated on a 10-15.5% gradient gel. Figure 3.6 clearly demonstrates that meristem and leaf protein band patterns are highly dissimilar and that the meristem lane contains a higher number of protein bands.

In total, 35 non-redundant proteins were identified (table 3.4).

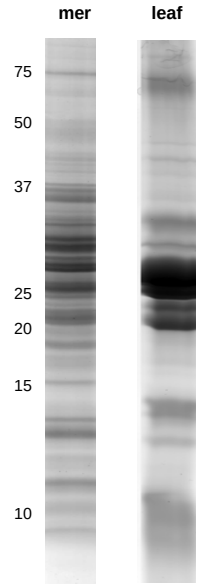


Figure 3.6: *Banana meristem (mer) and leaf (leaf) proteins soluble in a 5/4 C/M mixture separated on a 10-15.5% gradient gel and Coomassie Brilliant Blue stained. Molecular masses (kDa) of a protein standard are indicated on the left.*

Table 3.4: *Proteins identified after C/M extraction of a whole banana meristem lysate combined with one-dimensional gradient (1-D) SDS-PAGE. Swiss-Prot accession number, the closest protein homolog, protein score (Mascot), the NR per protein, the predicted physicochemical properties; relative molecular masses (M_r, × 1000), pI, the number of transmembrane helices (TMH, determined by Aramemnon), GRAVY scores and the location (CW= cell wall, CY= cytoplasm, EM= endomembrane system, M= mitochondrion, N= nucleus, PL= plastid, PM= plasma membrane, R= ribosome, V= vacuole, U= unknown, are shown.*

Accession	Closest homolog	Score	NR	M_r	p*I*	TMH	GRAVY	Location
A7Q777	Chromosome chr18 scaffold_59 [*Vitis vinifera*]	136	2	39.18	5.39	1	-0.153	CW
P38076	Cysteine synthase [*Triticum aestivum*]	133	1	34.11	5.48	0	0.07	CY
P48534	L-ascorbate peroxidase [*Pisum sativum*]	87	1	27.06	5.52	0	-0.332	CY
P29448	Thioredoxin H-type 1 [*Arabidopsis thaliana*]	279	3	12.67	5.64	0	0.034	CY
Q5JL11	Putative soluble inorganic pyrophosphatase [*Oryza sativa*]	206	3	23.62	5.88	0	-0.31	CY
O23714	Proteasome subunit beta type-2-A [*Arabidopsis thaliana*]	294	5	22.54	5.95	0	-0.034	CY
P34921	Glyceraldehyde-3-phosphate dehydrogenase [*Dianthus caryophyllus*]	117	1	36.9	6.46	0	-0.143	CY
Q9AYP4	40S ribosomal protein S10 [*Oryza sativa*]	92	3	20.26	9.76	0	-0.878	CY
Q8H0X6	Cysteine proteinase inhibitor 6 [*Arabidopsis thaliana*]	83	1	23.47	5.66	1	-0.353	EM
Q07078	Heat shock protein 81-3 [*Oryza sativa*]	81	1	80.18	4.98	0	-0.599	M
Q9FWR4	Glutathione S-transferase DHAR1 [*Arabidopsis thaliana*]	117	1	23.64	5.56	0	-0.173	M
P47922	Nucleoside diphosphate kinase 1 [*Pisum sativum*]	67	1	16.46	5.94	0	-0.064	M
P17783	Malate dehydrogenase [*Citrullus lanatus*]	90	1	33.24	6.26	0	0.135	M
P27084	Superoxide dismutase [Mn] [*Pisum sativum*]	86	2	25.82	7.16	0	-0.269	M
Q6K548	Mitochondrial outer membrane protein porin [*Oryza sativa*]	94	2	29.09	7.21	18 β	-0.16	M
O48646	Probable phospholipid hydroperoxide glutathione peroxidase 6 [*Arabidopsis thaliana*]	215	4	19.61	7.85	0	-0.277	M
A5AHP2	Chromosome chr15 scaffold_37 [*Vitis vinifera*]	78	1	31.95	9.61	1	-0.124	M
Q2PF08	ADP,ATP carrier protein 1 [*Trifolium pratense*]	197	2	39.9	9.84	3	0.007	M
Q9LT08	26S proteasome non-ATPase regulatory subunit 14 [*Arabidopsis thaliana*]	118	2	34.35	6.31	0	-0.243	N
A7PF22	Chromosome chr11 scaffold_13 [*Vitis vinifera*]	176	2	15.69	6.1	1	0.113	N-CY
Q7XLR1	Probable aquaporin PIP2-6 [*Oryza sativa*]	108	1	29.96	9.08	5	0.483	PM
P04907	Glutathione S-transferase 3 [*Zea mays*]	128	2	23.72	6.06	0	0.066	PL
Q08682	40S ribosomal protein Sa-1 [*Arabidopsis thaliana*]	179	3	32.29	5.05	0	-0.316	R
P59263	Ubiquitin [*Arabidopsis thaliana*]	185	2	8.52	6.56	0	-0.445	R
B9IJE3	Predicted protein [*Populus trichocarpa*]	121	1	35.87	5.55	0	-0.016	U
P49036	Sucrose synthase 2 [*Zea mays*]	69	2	92.94	6.03	0	-0.282	U
P52578	Isoflavone reductase homolog [*Solanum tuberosum*]	236	3	33.85	6.16	0	-0.063	U
Q6L4X6	Os05g0508400 protein [*Oryza sativa*]	270	2	66.32	7	0	-0.448	U
A9PIA4	Predicted protein-Putative uncharacterized protein [*Populus trichocarpa*]	128	1	24.28	7.02	0	-0.028	U
Q0DIK0	Os05g0383000 [*Oryza sativa*]	130	2	17.89	7.7	1	-0.222	U
Q39613	Peptidyl-prolyl cis-trans isomerase [*Catharanthus roseus*]	193	3	18.28	8.36	0	-0.27	U
Q0J8X2	Os04g0683100 protein [*Oryza sativa*]	123	2	29.1	9.82	0	-0.392	U
P21616	Pyrophosphate-energized vacuolar membrane proton pump [*Phaseolus aureus*]	80	1	79.85	5.32	15	0.647	V
P24091	Endochitinase B [*Nicotiana tabacum*]	74	1	31.47	8.31	0	-0.322	V
P07979	Lichenase [*Nicotiana plumbaginifolia*]	122	2	37.48	9.63	1	-0.128	V

As could be expected, protein identifications from the meristem cells differed from those found in the leaf extract except for the mitochondrial form of malate dehydrogenase. Only the most abundant proteins could be identified. Meristematic cells are fast dividing cells, associated with a high level of protein synthesis and consequently a high abundance of ribosomal proteins. Moreover, the expression of proteasome subunit genes is elevated since plant cell division is linked to a timely proteolysis of several cell cycle regulators and a rapid formation and removal of structures such as the spindle apparatus and phragmoplast (Kurepa and Smalle, 2008). This implies a high abundance of proteins that are involved in the ubiquitination process and explains the identification of ubiquitin, superoxide dismutase and proteasome subunits. Due to the presence of a high number of small vacuoles in meristem cells, vacuolar proteins such as endochitinase were detected. Also cyclophilin (peptidyl-prolyl cis-trans isomerase) is a highly abundant protein in meristematic tissue (Nuc et al., 2001).

Most proteins were located in mitochondria (figure 3.4C). However, the location of the identified proteins was more diverse in meristems in comparison with leaves (figure 3.4B). This is of no surprise since leaf cells are specialized in photosynthesis while meristem cells are still undifferentiated.

The number of identified membrane proteins was lower compared to leaves (29 %). Nevertheless, the pyrophosphate-energized vacuolar membrane proton pump which contains 15 TMH could be detected.

We hypothesize that also non-membrane proteins are extracted in a C/M mixture and that proteins soluble in C/M are able to associate with lipids or contain a short hydrophobic region as mentioned by Rolland et al. (2006). It was for example demonstrated that the cytosolic form of nucleoside diphosphate kinase associates with membranes of a wide variety of intracellular compartments in human (Mitchell et al., 2009). Also GAPDH was reported to associate with subcellular membranes in a rather unspecific way (Zinser and Daum, 1995). Mitochondrial and chloroplast proteins could still be associated with their transit peptide at the moment of C/M extraction. The monomeric form of malate dehydrogenase was shown to associate with phospholipid vesicles, whereas the native dimer did not (Webster et al., 1980). Also the phospholipid hydroperoxide glutathione peroxidase is able to bind phospholipids. Glutathione S-transferase contains a hydrophobic binding site because it links glutathione to a hydrophobic substrate to detoxify endo- and xenobiotic compounds (Neue-

feind et al., 1997). This hypothesis was also valid for some proteins without a predicted TMH identified in the C/M-soluble fraction of leaves. Aldolases are for example major components of chloroplast plastoglobules which are lipid-rich structures (Ytterberg et al., 2006). Also some proteins of the photosynthetic apparatus, like photosystem I psaH and Lhca4 protein, were detected in these globules. However, this is only a hypothesis which needs to be confirmed by experimental results.

3.3.3 Chloroform/methanol extraction on whole cell lysates: evaluation of the method

To estimate the value of chloroform/methanol extraction as a method to study membrane proteins from total cellular lysates, a comparison was made with reports that utilized the same C/M ratio (i.e. 5/4) to study membrane proteins from purified membrane structures. A first observation was that C/M extraction is reproducible. Henriques and Park reported in 1976 that more than 50% of the total C/M-soluble proteins of spinach chloroplasts are present in a 25 kDa band (Henriques and Park, 1976). This band was specified further as containing the main component of the light-harvesting chlorophyll-protein complex (LHC). The most abundant proteins found in the C/M fraction of *Arabidopsis* and banana leaves are indeed also associated with LHC II and have a molecular mass of approximately 25 kDa (figures 3.1, 3.2 and 3.3). Subsequently, we compared the proteins that we identified in the C/M-soluble fraction of *Arabidopsis* leaves (table 3.2) with those identified in C/M extracts of chloroplasts by other researchers. The majority of the 36 identified proteins (28) were previously reported, 22 of them in the study of the thylakoid proteome by Friso et al. (2004) (table A.3). The other 8 proteins were not previously reported to be C/M-soluble, but were not identified as membrane proteins. This indicates that a mixture of chloroform and methanol extracts a higher number of non-membrane proteins from a total cellular lysate compared to purified membrane structures.

To make a final conclusion, we calculated the number of membrane proteins identified in the different studies.

Table 3.5 shows that C/M extraction is more suitable for membrane proteome studies on chloroplasts compared to other organelles. A possible explanation is the presence of highly abundant membrane proteins in chloroplasts compared to other membrane structures from which the proteome is more diverse (e.g. the plasma membrane). The higher number reported by some authors can also be partly explained by the less stringent criteria HMMTOPv2.0 uses for prediction of TMH. Indeed, in our strategy the program predicted 72% of the C/M extracted proteins from *Arabidopsis* leaves listed in table 3.2 to be transmembrane proteins. Extracts from total cellular lysates contain less membrane proteins in comparison with extracts from purified organelles. In studies on *Arabidopsis* chloroplasts 42 of 53 (Ferro et al., 2002) and 34 of 37 (Ferro et al., 2003) extracted proteins were identified as integral membrane proteins. In our total leaf extract 21 of 36 proteins were predicted to contain at least one transmembrane helix. This lower ratio can be explained by the presence of abundant non-membrane proteins in the total extract which are removed through purification of the organelle. The lower number of identified membrane proteins from meristem samples can be explained by its more diverse proteome with a smaller number of abundant membrane proteins.

Table 3.5: *Comparison of the number of membrane proteins identified after chloroform/methanol (5/4) extraction of different samples. The tissue where the extraction was performed on, the method to separate the proteins (geLC: combination of SDS-PAGE and liquid chromatography), the prediction program for TMH and the number of identified proteins (total non-redundant (NR), membrane, and non-membrane proteins) are shown. The % membrane proteins was calculated based on these numbers.*

Organelle	Separation method	TMH prediction	NR proteins	membrane	non-membrane	% membrane
Spinach chloroplast (envelope)[12]	1-D SDS-PAGE	HMMTOP	53	42	11	79
Arabidopsis chloroplast (envelope)[13]	geLC	HMMTOP	37	34	3	92
Arabidopsis mitochondria [14]	geLC	HMMTOP	31	22	9	71
Arabidopsis plasma membrane[15]	1-D SDS-PAGE and geLC	Aramemnon	59	32	27	54
Cauliflower vacuoles (tonoplast) pH 4[16]	geLC	Aramemnon	43	27	16	63
Cauliflower vacuoles (tonoplast) pH 6	geLC	Aramemnon	30	19	11	63
Arabidopsis total leaf	1-D and dSDS-PAGE	Aramemnon	36	21	17	58
Banana total leaf	1-D and dSDS-PAGE	Aramemnon	20	14	6	70
Banana total meristems	1-D SDS-PAGE	Aramemnon	35	10	25	29

[12] Ferro et al. (2002)
[13] Ferro et al. (2003)
[14] Brugière et al. (2004)
[15] Marmagne et al. (2004)
[16] Schmidt et al. (2007)

3.4 Conclusions and future direction

From the results presented in this chapter we conclude that a mixture of chloroform and methanol selectively extracts a limited number of proteins in a reproducible way. For the first time, membrane proteins with more than 2 TMH from meristems were reported. The advantage of performing a C/M extraction is that complexity of the sample is highly reduced which allows protein separation through 1-D SDS-PAGE. The drawback is that less proteins are identified compared to other techniques for membrane proteomics. Moreover, like Bräutigam et al. (2008), we could not demonstrate a strong correlation between the hydrophobicity index nor the number of predicted membrane helices and solubility in the organic solvents. We therefore hypothesize that the method selectively extracts proteins that are associated with certain lipids at the moment of extraction but this hypothesis needs further experimental evidence. For protein separation, a one dimensional separation using an optimized acrylamide gradient is recommended since the dSDS yielded only a limited number of additional identifications and is more labour intensive. However, the application of a geLC approach could result in more identifications because of an improved resolution. This strategy should be considered for future studies in case an initial estimation of the number of proteins per band indicates that this number is sufficiently high. Since C/M extraction proved to complement classical 2-DE studies, we believe that the method can be a valuable tool in membrane proteomics studies in poorly sequenced plants, but only in combination with other techniques for membrane proteomics.

This chapter is based on the paper: Vertommen, A., Panis, B., Swennen, R. and Carpentier, S. C. 2010. Evaluation of chloroform/methanol extraction to facilitate the study of membrane proteins of poorly sequenced plants. *Planta* 231 (5), 1113-1125

Chapter 4

Evaluation of Blue Native electrophoresis as a method to enrich samples in membrane protein complexes

In all science, error precedes the truth, and it is better it should go first than last.

Hugh Walpole

4.1 Introduction

In the previous chapter we looked for a technique to selectively extract membrane proteins from a total cellular fraction keeping in mind (i) that meristem tissue is only available in very small amounts and (ii) that no prior protein selection is wanted. We concluded that the mixture of chloroform and methanol only extracts a limited number of membrane proteins and that it therefore should be

complemented with other techniques suitable for membrane proteomics. One of these techniques is blue native electrophoresis (see chapter 2, section 2.4). With this method proteins that belong to a protein complex are separated from the bulk of proteins, which simplifies the protein pool to be analyzed. This is a prerequisite to handle the low abundance of membrane proteins. Moreover, as explained in figure 2.4 on page 50, also the individual subunits belonging to one complex can be separated from each other using BN-SDS-PAGE (or BNE). Since the chance that only one protein is present in one spot of the second dimension gel is high, BNE is suitable for membrane proteomics in non-model plants (see figure 1.5). In addition, we preferred blue native electrophoresis above alternative techniques described in chapter 2 because we were also interested in the study of protein-protein interactions. Knowledge of these interactions is a key step in the integration of proteomics data in a systems biology approach. Other advantages of this technique are that no expensive or highly sophisticated equipment is required and that protein complexes which are composed of very heterogenic proteins can be analyzed.

Briefly, blue native electrophoresis comprises the following steps; (i) isolation of protein complexes in the presence of a mild, non-ionic detergent (ii) addition of the dye Coomassie Brilliant Blue (CBB) G250 to provide a negative charge and (iii) separation of protein complexes under native conditions (i.e., 4 ℃, no denaturating agentia) according to their molecular size. In its most popular application (i.e., BNE), it is combined with denaturating SDS-PAGE to separate and visualize individual subunits belonging to the same complex (see figure 2.4). Blue native electrophoresis was originally developed to study bovine mitochondrial proteins and complexes by Schägger and von Jagow (1991) and gained popularity during the next two decades. In these years, the protocol has only slightly been adapted which demonstrates its robustness. The technique is suitable for the separation of soluble and membrane protein complexes but is typically used for the study of membrane protein complexes. The main disadvantages of BNE are its poor performance in quantitative studies and its relatively low resolution and dynamic range (Miernyk and Thelen, 2008). A problem for quantitative BNE studies lies in the need to manually excise the first dimension lane and the transfer from the first to the second dimension which is quantitatively poorly reproducible. As a solution, the DIGE technology was proposed (Heinemeyer et al., 2009). Using Cydyes, two or three samples can be run in the same lane and will thus undergo the same excision

and transfer. Moreover, it is a very sensitive staining technique which allows visualization of less abundant subunits. A second alternative is the application of a geLC approach were one dimensional BN is combined with LC-MS/MS and label-free quantification as proposed by Wessels et al. (2009). In plants, BNE is mainly applied to study the mitochondrial respiratory and chloroplast photophosphorylation complexes as reviewed in Eubel et al. (2005). Only one plant report describes protein complexes in total cellular lysates (Remmerie et al., 2009).

In this chapter, we evaluate the use of BN in banana. In an initial experiment, protein complexes in total cellular lysates and total membrane fractions are compared to demonstrate the enrichment of membrane protein complexes in the latter. Subsequently, proteins identified in protein complexes of total membrane fractions of banana leaves and meristems are discussed to estimate the value of the BNE regarding its capacity to (i) analyze membrane proteins and (ii) study protein-protein interactions. In addition, a BN-LC approach is evaluated for its use in non-model plants as this approach avoids the protein loss associated with the transfer from the first to the second dimension. For this evaluation, leaf membrane fractions were preferred as starting material because they have already been extensively studied in literature and because of the availability of large amounts of starting material.

4.2 Materials and methods

4.2.1 Plant material and isolation of the protein complexes

Banana plants (Cachaco cultivar, ITC 0643) were received from the Bioversity International Transit Centre (ITC) and meristem cultures were initiated as described by Strosse et al. (2006). The meristem cultures were maintained in the dark at 25 °C. One month after subculture, meristems were excised from the meristem clump (total: 2g), crushed in liquid nitrogen and added to an extraction buffer containing 100 mM Tris (pH 8.3 with HCl), 5 mM EDTA, 2% DTT, complete protease inhibitor cocktail (Roche) and 0.33 M sucrose in a ratio 1 : 3 (sample : buffer). After vortexing for 5 min at 4 °C, nuclei and cell

debris were removed by centrifugation at 6500 *g* for 10 min. Supernatans was added to cold ultracentrifugation tubes and a membrane pellet was obtained after centrifugation at 100 000 *g* for 1.5 h. Complexes from total cellular lysates were solubilised as described by Remmerie et al. (2009). Membrane pellets were resuspended by vortexing in 50 µL of a buffer containing 30 mM Hepes (pH 7.4), 150 mM potassium acetate and 30% glycerol. For labeling with CyDyes, the pH was adjusted to pH 8.5 (with KOH). For the DIGE approach, samples were randomly labelled with Cy-3 and Cy-5 dyes before protein separation. An internal standard, which is a mixture of equal amounts of each sample, was labeled with Cy-2. The samples were randomly grouped and mixed with the Cy-2 labeled internal standard. All steps were performed as cold as possible to retain the native composition of the protein complexes. The protein concentration was determined using a modified micro-Bradford protein assay (Zuo and Lundahl, 2000).

4.2.2 Protein separation

4.2.2.1 First dimension: BN-PAGE

Protein complexes were solubilized by adding 2% digitonin (approximately 8.5 µg per µg protein). After incubation for 30 min on ice, samples were centrifuged for 30 min at 16 000 *g* to remove insoluble material. After adding 1/10 volume loading buffer (500 mM aminocaproic acid, 2% Serva blue G), samples were loaded onto the first dimension polyacrylamide gel (5-16%, linear gradient casted with the 2DE optimizer (NextGen Sciences) and a 4% stacking gel (24 cm × 0.75 mm)). Electrophoresis was carried out at 0 ℃ at 2 W per gel for 12 h 30 min. The cathodic buffer was composed of 50 mM Tricine, 15 mM BisTris and 0.02% Coomassie blue (Serva) (pH 7.0). The anodic buffer contained 50 mM BisTris. Both buffers were stored at 4 ℃ before use. The blue cathodic buffer was replaced by a colourless buffer (the same buffer without the CBB) after two third of the running time.

4.2.2.2 Second dimension: SDS-PAGE

Individual lanes (5 mm) from the first dimension gel were excised and equilibrated for 30 min in a buffer containing 3% SDS, 66 mM Na_2CO_3, 1% DTT, 30% glycerol and 50 mM Tris-HCl, pH 6.8. Individual lanes were placed on top

of second dimension polyacrylamide gels (12.5% with 4% stacking, 20 cm × 1.00 mm) and sealed with 0.5% agarose. Separation was carried out overnight at 20 ℃ at 2 W per gel. After electrophoresis, gels were stained with CBB and stained spots were manually excised. After washing with 50 mM ammoniumbicarbonaat and 50% acetonitrile, trypsin (dissolved in 25 mM AmBic, 10% ACN) was added and proteins were digested overnight at 37 ℃. The resulting peptides were spotted on disposable MALDI-TOF target plates as described in the previous chapter.

4.2.2.3 Second dimension: LC

In the LC approach, the first dimension gel was stained with CBB. Stained bands were excised and divided in small gel pieces (1 mm^2) to facilitate tryptic digestion and peptide extraction. Tryptic digestion was performed as described above. Peptides were separated and analyzed using LC-ESI-QTRAP MS/MS (CapLC, Waters; QTRAP 2000, Applied Biosystems). Peptides were trapped on a C18 trapping column which was switched in line with a C18 analytical column. Peptide elution occurred by using an organic solvent gradient (5 - 95% of solvent B) (A: 0.1% FA, 1% ACN, B: 95% ACN, 0.1% FA) and directly sprayed into the mass spectrometer for peptide fragmentation analysis.

4.2.3 Protein analysis and identification

Mass spectrometry and identification was performed at CeProMa[1]. Peptides originating from the 2-D gel spots were analyzed on a 4800 MALDI TOF-TOF (ABI) in positive electron mode and externally calibrated. Their Mascot server was used for searches against the *Viridiplantae* (NCBI and Swiss-Prot) and the *Musa* EST databases. Carbamidomethylation of Cys was set as fixed and oxidation of Met as variable modification. The searching parameters allowed 1 missed cleavage, a peptide mass tolerance of 40 ppm and a fragment mass tolerance of 0.2 Da. The same criteria as described in the previous chapter were used to evaluate the identifications from the second dimension gel (i.e., the use of the MOWSE score and decoy database searches). Proteins identified by the LC approach were estimated as significant when at least one ion score and the protein score (Mowse score) were significant.

[1] `http://www.ceproma.ua.ac.be/`

The characterization of the identified proteins was performed in the same way as described in the previous chapter.

4.3 Results and discussion

4.3.1 The choice of detergent

As mentioned in section 2.4 in chapter 2, the choice of the detergent is an important factor for complex solubilization. In our study, digitonin was preferred since this detergent stably retains protein-protein interactions in a wide concentration range. We compared the performance of different digitonin concentrations (1, 2, 3, 4, 6 and 8%) and determined the concentration yielding the most intense bands (i.e., 2 %; not shown). The other two frequently applied non-ionic detergents, Triton and n-dodecyl-b-D-maltoside, are more sensitive to concentration changes as illustrated by Eubel et al. (2004). Only at a specific detergent/protein concentration, which has to be determined for every complex, they can dissolve the intact complex (Reisinger and Eichacker, 2008). The explanation can be found in the way the different detergents work. Digitonin disrupts protein-lipid interactions involving so-called "annular lipids" (Hunte, 2005). These are lipids which surround protein complexes on the outside and mediate the contact between the lipid bilayer and the protein complex. In a way digitonin excises a "piece of pie containing the complex" from the membrane structure (L. Eichacker, personal communication). When the concentration of digitonin is raised, the same pie will be excised, only now more detergent molecules will enter the membrane ("the knife is sharper"). Digitonin is therefore preferred for the study of supercomplexes. The other two detergents break interactions with "non-annular surface lipids". They bind at specific positions on the surface of a protein which are typically situated between the different subcomplexes. By raising the concentration of detergent, these interactions will be broken and the complex will dissociate in subcomplexes. Excessive detergent concentrations will lead to protein aggregation by stripping away essential bound lipids from the protein surface (Prive, 2007).

4.3.2 Enrichment in membrane proteins; total versus membrane complexes

Using the optimized concentration of digitonin, we compared complexes present in a total cellular fraction (figure 4.1A) and a total membrane fraction (figure 4.1B) of banana meristems to evaluate the enrichment in membrane protein complexes in the latter.

This figure also allows us to demonstrate some disadvantages of BNE. Monomeric proteins not belonging to a complex are separated in a hyperbole. Also single proteins and protein complexes smaller than 100 kDa are not well resolved due to the high abundance of proteins in that size range and the limiting separation distance as a consequence of the used acrylamide gradient. In analogy with the C/M extraction, BN is thus also restricted to the study of a specific subset of proteins. Only the proteins with a high M_r and proteins belonging to large protein complexes can be analyzed properly.

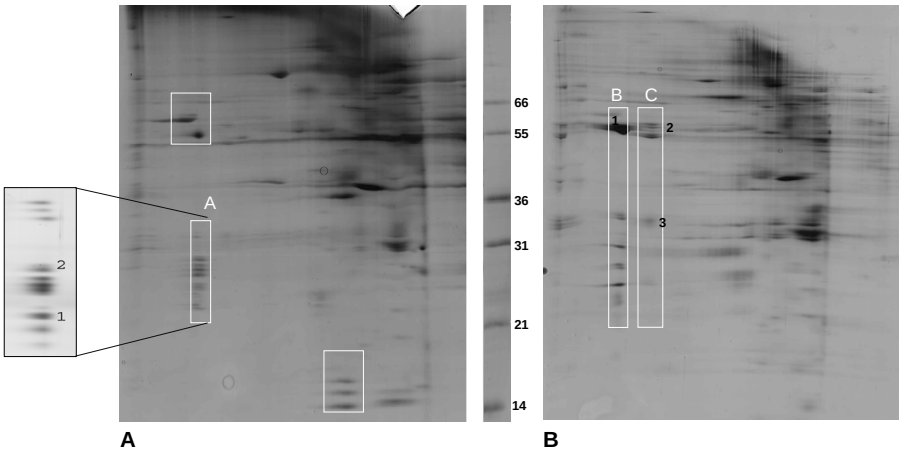


Figure 4.1: *Comparison of protein patterns after BNE of a total cellular fraction (A) and membrane fraction (B) of banana meristems. Proteins (80 µg) were separated on 18 cm × 0.75 mm gels in the first dimension and 20 cm × 1 mm in the second dimension. Gels were silverstained. The rectangles indicate the protein patterns that are prominent in each fraction. The magnification shows the proteasome complex (from a different, CBB stained gel). The letters and numbers refer to the protein patterns and proteins listed in table 4.1. A molecular mass standard is shown in the middle.*

Table 4.1: *An overview of the proteins identified in the spots numbered on figure 4.1. The complex, the number of the spot, the closest homolog, protein score (Mascot) and the number of non-redundant peptides that leaded to the identification (NR) are listed.*

Complex	Spot	Closest homolog	Score	NR
A	1	20S proteasome beta subunit 5 [*Citrus maxima*]	83	3
	2	Proteasome subunit alpha type-7 [*Bos taurus*]	71	2
B	1	ATP synthase subunit beta, mitochondrial [*Nicotiana plumbaginifolia*]	113	6
C	2	Probable mit.-processing peptidase subunit beta [*Arabidopsis thaliana*]	113	7
	3	Cytochrome c1-2, heme protein, mitochondrial [*Solanum tuberosum*]	75	3

By comparing the protein patterns (rectangles) on figure 4.1, clear differences between the total cellular lysate (figure 4.1A) and membrane fraction (figure 4.1B) gels could be distinguished. However, both gels also show similarities, probably explained by the presence of the same, highly abundant proteins in both fractions.

Some representative proteins were identified and protein patterns were compared with protein complexes described in literature. The most prominent complex in the total cellular fraction was recognized as the 20S core particle of the 26S proteasome complex (complex A, table 4.1). The protein pattern of this complex is very recognizable with most subunits having a molecular mass around 25 kDa. Roelofs et al. (2009) recently demonstrated the evolutionary conservation of the proteasome assembly which explains why this pattern is easy recognizable. Other papers confirm the presence of this complex in total cellular lysates (Camacho-Carvajal et al., 2004; Remmerie et al., 2009; Swamy et al., 2009). The membrane fraction on the other hand, was enriched in mitochondrial complexes (complex B, C table 4.1 and similarity with mitochondrial complex protein patterns (e.g., Jänsch et al. (1996)). This confirms that soluble complexes were depleted from the membrane fraction. We further focused on the analysis of the protein complexes in the membrane fraction.

4.3.3 Protein complexes in banana leaf and meristem membrane fractions

For the analysis of protein complexes present in banana leaf and meristem membrane fractions, resolution was increased through the use of longer first dimension gels (i.e., 24 cm instead of 18 cm). The reproducibility of the complex separation was estimated by comparing biological replicates and was considered sufficiently (figure 4.2).

We evaluated whether BNE is an appropriate technique for (i) the study of membrane proteins and/or (ii) the study of protein-protein interactions in a non-model plant.

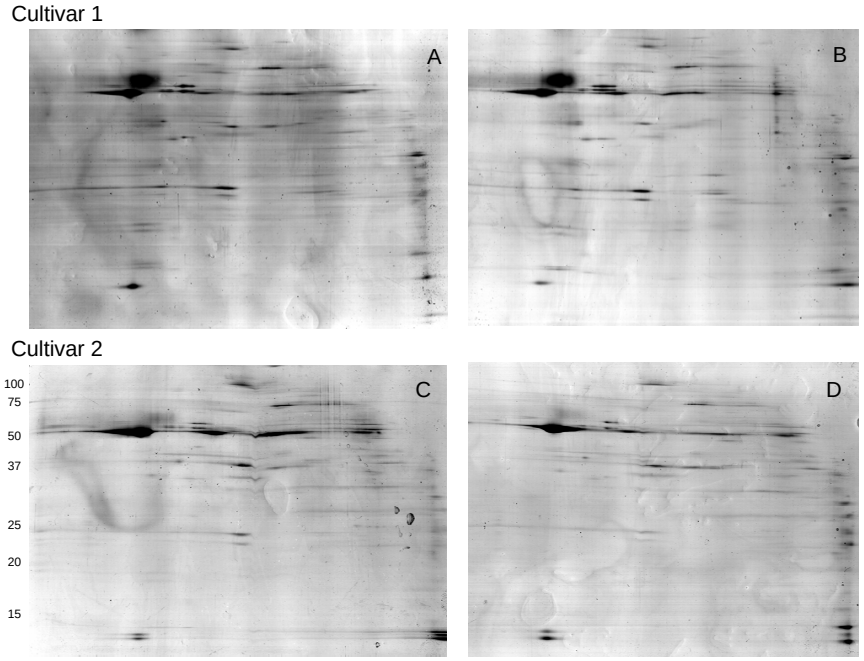


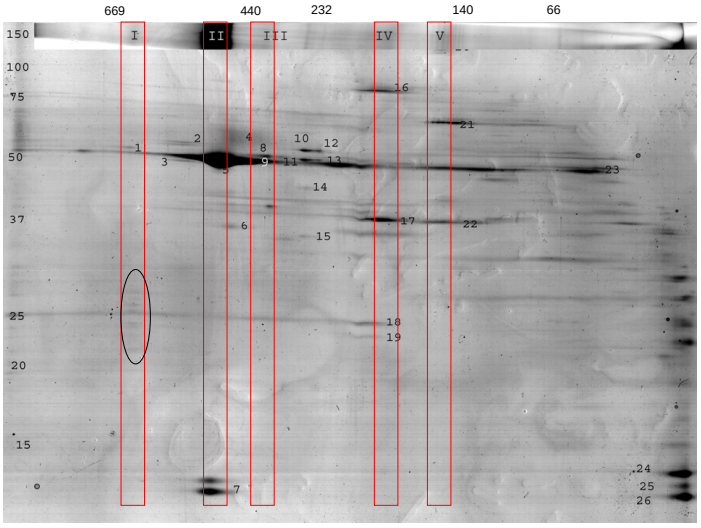
Figure 4.2: *Two biological replicates of BNE gels of membrane fractions of leaves of two banana cultivars are shown (CBB stained gels). Although differences between the different gel images exist, the BN technique appears to be rather reproducible.*

4.3.3.1 Blue native electrophoresis evaluated for the study of membrane proteins

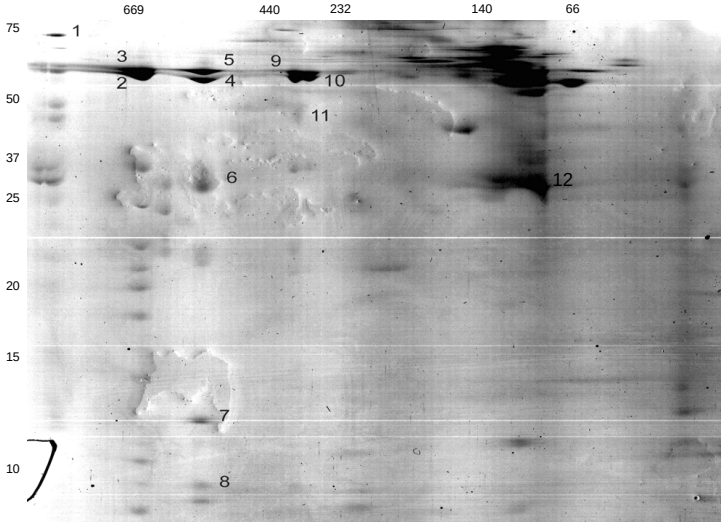
To evaluate BN as a method to study membrane proteins, proteins from a leaf (figure 4.3A) and meristem (figure 4.3B) gel were identified (tables 4.2 and 4.3 respectively).

The protein identification rate was rather low (around 40 %) because the S/N ratio of most peptide ions was too low. Possible reasons for this low S/N ratio are a low protein abundance, probably in combination with a poor digestion, peptide extraction and/or ionization. A low protein abundance is likely since more sensitive stains (e.g., Cydye staining) visualized more subunits per complex (figure 4.4). This low abundance might be a consequence of the mild solubilization conditions which are required to keep protein complexes intact. Since blue aggregates were observed in the first dimension wells, it is likely that the CBB concentration was too low or the salt concentration too high to prevent protein aggregation. Reducing the used salt concentration to half the amount, however, did not result in a significant improvement (not shown). Also the transfer of proteins from the first to the second dimension inevitably leads to a decrease in protein abundance.

Another explanation for the low identification rate is that proteins were still associated with lipids because of the mild solubilization conditions. This results in a poor tryptic digestion and recovery of peptides from the gel. A possible solution could be to perform a digestion in the presence of methanol and to add chloroform to remove the lipids as proposed by Mitra et al. (2007). This method will be used in the next chapter.



A. Leaves



B. Meristems

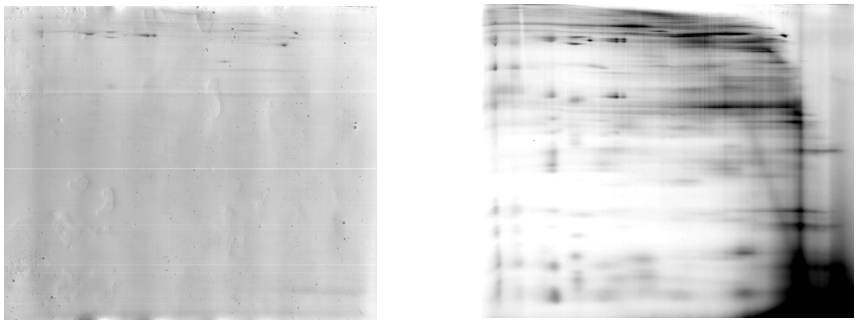
Figure 4.3: *Coomassie stained BNE gels of the membrane fraction (80 µg) of banana leaves (A) and meristems (B). The numbers on the second dimension gels refer to the proteins listed in table 4.2 and table 4.3 for leaf and meristem proteins respectively. The roman numbers on the first dimension gel lane (gel A) refer to the complexes listed in table 4.4. The black oval (gel A) indicates the 20S proteasome complex. The vertical lanes indicated by bars (gel A) indicate the protein subunits on the second dimension gel belonging to the band indicated on the first dimension gel. Molecular mass standards of the first dimension gels are shown on top and those of the second dimension gels are shown on the left (in kDa).*

Table 4.2: *Proteins identified in protein complexes of banana leaves separated by BNE (figure 4.3A). The procedure is described in section 4.2. The Swiss-Prot accession number, the identified protein homolog, the predicted M_r and pI, the protein score (Mascot), the NR identified per protein, the number of TMH and the predicted location (M=mitochondrion, C= chloroplast, S= secreted) are shown. The FDR was estimated 0 after a decoy database search.*

Spot	Accession	Homolog	M_r	pI	Score	NR	TMH	Location
1	P18260	ATP synthase subunit alpha	56	6.02	235	9	0	M
2	Q42290	Probable processing peptidase subunit beta	59	6.3	113	6	0	M
3	Q9ZU25	Probable processing peptidase subunit alpha	55	5.94	128	4	0	M
4	A4QJJ8	Photosystem I P700 chlorophyll a apoprotein A2	83	6.89	131	4	11	C
5	P25836	Rubisco large chain	52	6.33	574	14	0	C
6	Q9SJU4	Probable fructose-bisphosphate aldolase 1	43	6.18	79	3	0	C
7	O24045	Rubisco small chain	21	9.23	202	6	0	C
8	P83484	ATP synthase subunit beta-2, mitochondrial	60	6.18	103	6	0	M
9	P25836	Rubisco large chain	52	6.33	542	12	0	C
10	P08215	ATP synthase subunit alpha, chloroplastic	55	5.75	361	7	0	C
11	Q06FV3	ATP synthase subunit beta, chloroplastic	54	6.64	525	12	0	C
12	P08215	ATP synthase subunit alpha, chloroplastic	55	5.75	361	7	0	C
13	Q06FV3	ATP synthase subunit beta, chloroplastic	54	6.64	525	12	0	C
14	Q5Z8V9	Delta-aminolevulinic acid dehydratase, chloroplastic	47	5.81	72	3	0	C
15	Q2LGZ2	ATP synthase subunit gamma, chloroplastic	13	4.64	189	2	0	C
16	P26969	Glycine dehydrogenase [decarboxylating], Mitochondrial	115	7.17	77	4	0	M
17	Q9LRR9	Probable peroxisomal (S)-2-hydroxy-acid oxidase	40	9.16	317	9	0	C
18	P08963	Chlorophyll a-b binding protein 2	28	5.69	227	4	3	C
19	P08963	Chlorophyll a-b binding protein 2	28	5.69	114	3	3	C
20	Q43155	Ferredoxin-dependent glutamate synthase	165	5.84	74	3	0	C
21	Q43848	Transketolase, chloroplastic	80	5.94	95	4	1	C
22	P17878	Glyceraldehyde-3-phosphate dehydrogenase	37	6.61	124	3	0	S/C
23	P25836	Rubisco large chain	52	6.33	691	16	0	C
24	Q41932	Oxygen-evolving enhancer protein 3-2	25	9.72	132	4	0	C
25	O24045	Rubisco small subunit	21	9.23	74	2	0	C
26	O24045	Rubisco small subunit	21	9.23	81	2	0	C
27	Q42623	Glutamine synthetase	39	5.28	78	1	0	C

Table 4.3: *Proteins identified in protein complexes of banana meristems (FDR after decoy database search = 0), separated by BNE. The spots numbered on the second dimension gel of figure 4.3B are listed. The protein accession number (Swiss-Prot or Musa database), the identified homolog, the predicted Mr and pI, the protein score (Mascot), the NR leading to the protein identification, the number of TMH and the predicted location (M=mitochondrion) are shown.*

Spot	Accession	Homolog	M_r	p*I*	Score	NR	TMH	Location
1	Q43644	NADH-ubiquinone oxidoreductase 75 kDa subunitl	80	5.87	181	4	0	M
2	P05493	ATP synthase subunit alpha	55	6.01	579	14	0	M
3	P17614	ATP synthase subunit betal	60	5.95	710	14	0	M
4	Q9ZU25	Probable processing peptidase subunit alpha	55	5.94	152	7	0	M
5	Q42290	Probable processing peptidase subunit beta	59	6.3	113	6	0	M
6	P29610	Cytochrome c1-2, heme protein, mitochondrial	29	5.29	75	3	2	M
7	Q9SUU5	Ubiquinol-cytochrome C reductase complex 14 kDa protein, putative	15	9.73	113	3	0	M
8	MUSE477TF	Ubiquinol-cytochrome C reductase UQCRX/QCR9-like family protein	8	9.45	61	3	0	M
9	P17614	ATP synthase subunit beta, mitochondrial	60	5.95	957	16	0	M
10	P05493	ATP synthase subunit alpha, mitochondrial	55	6.01	334	10	0	M
11	P93306	NADH-ubiquinone oxidoreductase 49 kDa subunit	46	6.68	84	6	0	M
12	Q9FKM2	porin like protein	13	7.98	95	4	15 beta	M



A B

Figure 4.4: *The same gel was stained with CBB (A) and Cydyes (B) to demonstrate the low protein abundance on the BNE gels. An amount of 40 µg of complexes present in membrane fractions of banana meristems was loaded.*

From tables 4.2 and 4.3 we could conclude that the current BNE protocol is not as successful as expected for the study of membrane proteins. On both gels, approximately 18 % (7 of 40) of the identified proteins were predicted to contain at least one TMH. This might be explained by the mild solubilization conditions that are needed to keep protein complexes intact. Moreover, trypsin was used for protein digestion which is known to lead to a poor recovery of peptides derived from transmembrane regions. Additionally, proteins without TMH that also belong to a membrane protein complex are solubilized more easily because they are not associated with membrane lipids. Moreover, sometimes a complex is composed of several copies of the same subunit (e.g., the ATPase complex consists of three copies of the soluble alpha and beta subunits). Generally soluble proteins are more abundant and generate a lot of suitable tryptic peptides which facilitates their identification. Finally, although our starting material was a total membrane fraction, contamination by abundant soluble complexes could not be avoided (e.g. the Rubisco complex on the leaf gel). Nevertheless, BNE has still potential to be a valuable tool for the study of protein-protein interactions in banana. Therefore, we took a closer look at the identified proteins.

4.3.3.2 Blue native electrophoresis evaluated for the study of protein-protein interactions

In the gel plugs picked from the meristem gel (figure 4.3B) only mitochondrial proteins were identified (table 4.3). Except for the porin, all identified proteins belong to mitochondrial oxidative phosphorylation (OXPHOS) complexes. The OXPHOS system forms the basis for mitochondrial ATP production. In most organisms it is composed of the ATP synthase complex (complex V) and four oxidoreductase complexes: the NADH dehydrogenase (complex I), the succinate dehydrogenase (complex II), the cytochrome c reductase (complex III) and the cytochrome c oxidase (complex IV) complex (figure 4.5) (Jänsch et al., 1996; Eubel et al., 2003; Nübel et al., 2009). On our gel, subunits of complex II and IV were not identified. This could be due to (i) the possible lower abundance compared to the other three complexes, (ii) an acrylamide gradient that was not optimal for their separation or (iii) a digitonin concentration that was too low for solubilization. The total F_OF_1 ATPase (figure 4.3B, spot 2,3 and probably also the spots below) is an integral membrane protein complex (appr. 580 kDa) that consists of two parts with different physicochemical and functional properties. The hydrophilic part (the F_1 part) is the largest with a M_r of appoximately 350. It consists of at least five subunits: α_3, β_3, γ, δ and ϵ and is responsible for the synthesis of ATP from ADP and inorganic

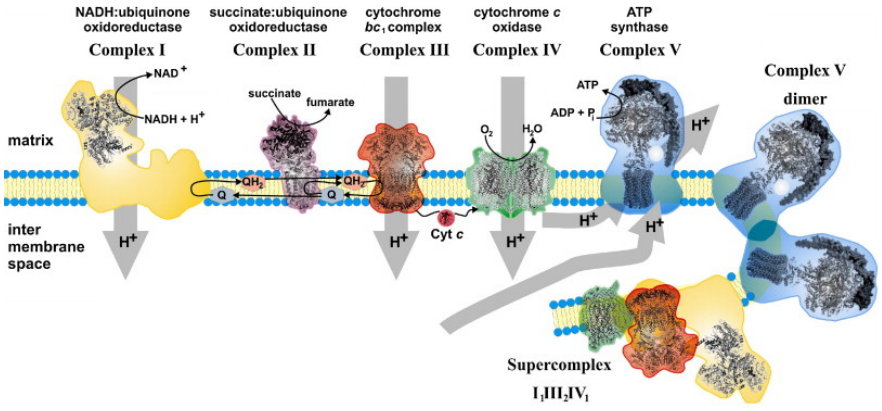


Figure 4.5: *Complexes in the oxidative phosphorylation (OXPHOS) chain. Adapted from Seelert et al. (2009)*

phosphate. The integral membrane part, F_O, is smaller (some 230 kDa) and is composed of at least seven subunits but is not well characterized in plants. None of the F_O subunits could be identified on our gel, probably because of their hydrophobic nature, low abundance (only one copy of each subunit) and lower molecular mass. However, based on the comparison with the BN pattern of the *Arabidopsis* (Heazlewood et al., 2003b; Eubel et al., 2003) and rice (Heazlewood et al., 2003a) mitochondrial F_OF_1 ATPase we can conclude that also the F_O subunits are present on our gel (figure 4.6).

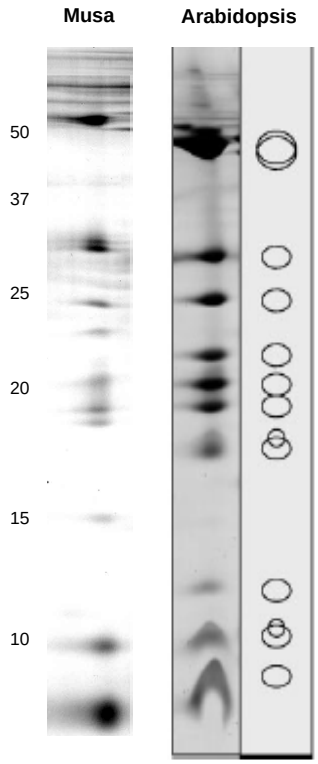


Figure 4.6: *A comparison of the BN pattern of complex V from banana meristems (left) and Arabidopsis cell suspension cultures (right) shows the similiarity between both patterns. The Musa picture is derived from a silverstained BNE gel of the membrane fraction of meristems (complete gel not shown); the Arabidopsis picture is adapted from Eubel et al. (2003) and shows the gel image as well as the schematic representation of the stained spots. Since the F_O part is not fully characterized, even in Arabidopsis, the identity of the individual subunits could not be shown. A molecular mass standard is indicated on the left.*

The soluble F1 subunits were also detected at a lower M_r in the first dimension (spots 9 and 10 on gel 4.3B) which is indicative for a dissociation of the whole F_OF_1 ATPase complex caused by the relatively high concentration of CBB dye in the cathodic buffer (i.e., 0.02%). Only at ten times lower dye concentrations of 0.002% (Krause, 2006) or by using clear-native PAGE (Wittig et al., 2006) the F_OF_1 ATPase remains intact. The higher concentration of dye was preferred since it is said to enhance the recovery of membrane proteins by preventing protein aggregation. According to Ma and Xia (2008) a sufficient amount of dye in the cathodic buffer is important to cover the entire surface of a protein with negative charges during electrophoresis. Regarding this dissociation, the subunits of the F_O part were also expected, but could not be identified.

Because digitonin was used, supercomplexes could be solubilized. Supercomplexes in mitochondria (i) enhance electron transfer rates because two complexes are physically connected thereby limiting the distance between them (ii) increase the stability of OXPHOS complexes, (iii) determine the ultrastructure of the inner mitochondrial membrane (i.e., the cristae), (iv) regulate respiration and (v) increase the protein insertion capacity of the inner mitochondrial membrane (Boekema and Braun, 2007; Nübel et al., 2009). The most abundant supercomplex is an association of complex I with complex III (on the left of figure 4.3B, spot 1 was identified as NADH ubiquinone oxidoreductase). The organization of these supercomplexes is explained in more detail by Bultema et al. (2008). On the leaf gel (figure 4.3A) the picture is more complicated.

Mainly soluble proteins from the cytosol, mitochondria and chloroplasts were identified. The most abundant complex from the cytosol, the proteasome complex (black oval), was recognized by the typical pattern of proteins around M_r 25 (see figure 4.1, magnification complex A). From the mitochondrial protein complexes we could identify the alpha and beta subunits of the F_OF_1 ATPase complex and the mitochondrial processing peptidase of the cytochrome C reductase complex. Most identified proteins were targeted to chloroplasts. The most abundant complex is the association of the large and small subunit of Rubisco. Its location at a high M_r is explained by its occurrence as a heterohexadecamer composed of 8 large and 8 small chains, in analogy with e.g., the enzyme of spinach (Knight et al., 1990). The monomeric subunits can be identi-

fied in the diagonal (e.g., spot 25 and 26, figure 4.3A). Two isoforms of the small subunit could be distinguished. Because of its high abundance, the Rubisco complex masks the presence of less abundant proteins. Subunits of thylakoid complexes were expected but only a small number of proteins involved in the photosynthesis process were identified. From the Photosystem (PS) I complex, the apoprotein A2 (spot 4, figure 4.3A) and subunit XI (spot 28, figure 4.3A) were identified. The apoprotein spot is very dispersed, probably because it contains 11 transmembrane domains. Therefore it is likely still associated with a large amount of lipids which results in a poor focusing. In contrast to PSI, no proteins belonging to PSII were identified. Photosystem II is located in the tightly appressed grana thylakoid membranes, while PSI resides in the non-appressed parts (Nelson and Ben-Shem, 2004). Because of the rigid structure of digitonin, the detergent is unable to efficiently interact with the densely appressed grana membranes. Therefore, it only extracts a small part of the PSII complexes compared to the PSI complexes which are easily accessible. We had already an indication for this partial solubilization of PSII complexes during the sample preparation step since the majority of the protein-bound chlorophylls were found in the pellet. Therefore, digitonin should be replaced by an alternative detergent such as n-dodecyl-b-D-maltoside or Triton X-100 when the aim is to study the PSII and LHCII complexes (Krause, 2006). Further improvements to enlarge the number of identified PS complex proteins in the future are the adjustment of the acrylamide concentration and/or the buffer system used. Several proteins belonging to the PS I and II complexes have a molecular mass ranging between 5 and 20. Therefore, an adjusted acrylamide concentration for the second dimension gel (e.g., 15%) in combination with the Tricine system (e.g., Kügler et al. (1997); Chen et al. (2007b)) is a better choice to achieve a sufficient separation of the low molecular mass proteins.

Since proteins from several organelles were identified, it is incorrect to claim that proteins detected on one vertical line always belong to the same complex. It also implies that no visual comparisons with other publications can be made since the separation pattern on figure 4.3A does not resemble published BNE gels of purified organelles. Moreover, high molecular weight proteins such as the ferredoxin-dependent glutamate synthase (spot nr. 20) co-migrated with small complexes. This enzyme has a M_r of 165 and is thus not associated with the protein spots below.

From these results we can conclude that total membrane fractions are too complicated to analyze protein-protein interactions using BNE. Additionally, only the most abundant subunits were visualized and identified as could be concluded from a comparison of the same gel stained by CBB and Cydyes (figure 4.4). Therefore we aimed at increasing protein abundance by avoiding the second dimension step where protein losses occur during the transfer from the first to the second dimension.

4.3.4 Evaluation of BN-LC

To avoid the uncontrollable protein loss associated with the transfer from the first to the second dimension and as such to increase protein abundance, we evaluated a BN based protein separation in combination with a LC based peptide separation. Because the use of BN restricts the number of proteins in one band to the proteins belonging to one complex, the complexity of the peptide pool that enters the mass spectrometer is significantly reduced which is beneficial for peptide based proteomics in poorly sequenced plants. Fandino et al. (2005) used this approach to analyze the composition of the OXPHOS complexes I, III, IV and V from bovine mitochondria. Also Wessels et al. (2009) proposed LC as alternative for SDS-PAGE in blue native analysis of protein complexes. The disadvantage of this technique is that the connectivity between the individual subunits can not be visualized. For our evaluation, total membrane fractions from leaves were used as starting material because of large amounts of this material were available. Total membrane fractions were preferred as starting material to make a comparison with the BNE results described above possible. From table 4.4 we could conclude that some identifications retrieved from the SDS gel (figure 4.3A) were confirmed and some new identifications were added.

Table 4.4: *Proteins identified in protein complexes of banana leaves by BN-LC. The numbers correspond to a band in the one-dimensional gel depicted on figure 4.3A, the identified proteins in one band (accession number and protein homolog), the predicted M_r and pI, the protein score (Mascot), the NR identified per protein, the number of TMH, the predicted location (M= mitochondrion, C= chloroplast, S= secreted) and the corresponding spots on the second dimension gel (BNE) are listed.*

Band	Accession	Homolog	M_r	p*I*	Score	NR	TMH	Location	BNE
I	B9T1V8	ATP synthase beta subunit, putative	60	6	167	3	0	M	/
	Q8SLZ4	Large chain Rubisco	49	6.33	149	4	0	C	/
	Q9LST5	Beta 5 subunit of 20S proteasome	30	5.72	137	2	0	S	oval
	A6N6L1	Catalase	34	6.8	112	3	0	S	/
	Q34265	F1-ATPase alpha subunit	46	7.89	95	2	0	M	1
	B7FMD3	Proteasome subunit (6) alpha type	27	5.83	93	2	0	S	oval
	PSB7B	Proteasome subunit beta type-7-B	24	6.23	61	1	0	S	oval
II	Q32272	Large chain Rubisco	49	6.33	475	17	0	C	5
	O24045	Small chain Rubisco	21	9.23	250	4	0	C	7
	P05311	Photosystem I P700 chl a apoprotein A2	83	7.11	106	2	11	C	4
	Q67H24	Photosystem II CP43 protein	46	6.55	82	1	6	C	/
III	A9QBI7	ATP synthase CF1 beta subunit, C	54	5.11	599	14	0	C	8
	A9QBN6	ATP synthase CF1 alpha subunit	55	5.32	414	9	0	C	/
	Q40257	Large chain Rubisco	37	7.37	151	3	0	C	9
	A9P1T4	ATP synthase gamma chain	42	8.57	66	1	0	C	/
IV	gi—228403	Glycolate oxidase	41	9.38	312	12	1	S	17
	A9PGW0	Fructose-bisphosphate aldolase	43	7.55	189	4	0	C	/
	B9U4P4	ATP synthase beta subunit	53	5.75	140	3	0	C	/
	P49361	P protein, glycine dehydrogenase	114	6.52	114	3	0	M	16
	O24045	Rubisco small subunit	21	9.23	89	4	0	C	/
	O49124	Putative serine-glyoxylate aminotransferase	44	7.63	82	1	0	S	/
V	P34921	Glyceraldehyde-3-phosphate dehydrogenase	37	6.46	231	5	0	S	22
	B9GPE7	Predicted protein (transketolase)	81	5.97	231	4	1	C	21
	Q8SLZ4	Rubisco large subunit	49	6.33	226	4	0	C	/
	P23225	Ferredoxin-dependent glutamate synthase1	177	6.21	216	7	0	C	/
	gi—10834738	NADP-dependent GAPDH A subunit	18	6.14	174	3	0	C	/

This approach looks thus promising for future applications. However, the method should be optimized further. Again, only the most abundant, mainly soluble proteins were identified and not all proteins that were detected on the SDS gel were identified. A probable cause is the presence of Rubisco in every band. This indicates either Rubisco is present in all those complexes; either Rubisco is smeared over the whole first dimension lane or a contamination during mass spectrometric analysis occurred. Because of the high abundance of Rubisco it is likely that this protein was unspecificaly associated with other proteins. In any case, the presence of Rubisco hindered the selection of peptides from less abundant proteins for fragmentation during MS/MS analysis because of its high abundance and its high number of tryptic peptides in the mass range of the mass spectrometer. Therefore, in future experiments an exclusion list should be made for Rubisco masses. Other improvements include the optimization of the peptide separation and the evaluation of alternative enzymes for protein digestion.

4.4 Conclusions and future direction

In the current chapter first insights in BN as a technique to study proteins belonging to membrane protein complexes in a poorly sequenced plant are provided. Since the results obtained in chapter 3 demonstrated that a total cellular fractions is too complex for membrane proteomics, we used a total membrane fraction as starting material. We could conclude that (i) also in the membrane fraction, contaminants were still present indicating that a more pure fraction is required as starting material, (ii) only abundant, mainly soluble proteins were identified which indicates that larger amounts of starting material are required, (iii) the detergent used is decisive for the isolation of a complex, (iv) high molecular weight proteins migrated with small complexes during the first dimension indicating that the interpretation of BNE gel patterns should be carefully evaluated and (v) the BN-LC combination looks promising but the identification of a higher number membrane proteins compared to the BNE approach was not yet demonstrated

However, we believe in the power of BN for the study of protein-protein interactions when there is no previous knowledge on bait proteins, but, further optimization is clearly required. These improvements should include (i) the use of isolated membranes as starting material (ii) the use of increased amounts of starting material (iii) the evaluation of alternative detergents for complex solubilization (iv) the adaptation of the salt concentration in the sample buffer to limit protein precipitation in the first dimension wells (v) the use of alternative enzymes (possibly in combination with trypsin) and (vi) the avoidance of contamination by Rubisco or other abundant proteins. Also the finding that shotgun proteomics can be applied on poorly sequenced plants in case the peptide pool that enters the mass spectrometer is of low complexity is really promising. This approach will not only be instrumental in our future BN studies where protein losses associated with the transfer from the first to the second dimension gel are avoided but also in other membrane proteome studies that focus on a specific organelle. Also this approach needs further optimization (e.g., the peptide separation). In conclusion, we recommend the use of BN to achieve a general overview of possible protein-protein interactions in a certain cell compartment. To obtain more detailed results, these observations should be complemented with other techniques using a protein of interest as bait protein.

Chapter 5

Focus on the plasma membrane

Science is wonderfully equipped to answer the question "How?" but it gets terribly confused when you ask the question "Why?"

Erwin Chargaff

5.1 Introduction

From the previous chapters we could conclude that a total membrane fraction is still too complex and not sufficiently pure for membrane proteome studies. In the current chapter we therefore focus on one specific membrane, the plasma membrane (PM). This membrane is surrounding all living cells and provides a barrier between the extra- and intracellular environment. Proteins associated with the PM membrane mediate the exchange of signals, nutrients and chemicals between both environments and are the starting point of many physiological processes. Therefore the study of the PM proteome will be instrumental in the study of the response of banana plants to environmental stresses. The PM proteome is very diverse because of the wide diversity of functions the PM proteins execute. Plasma membrane proteins are involved in transport, signaling, cell wall formation and cellular traffic.

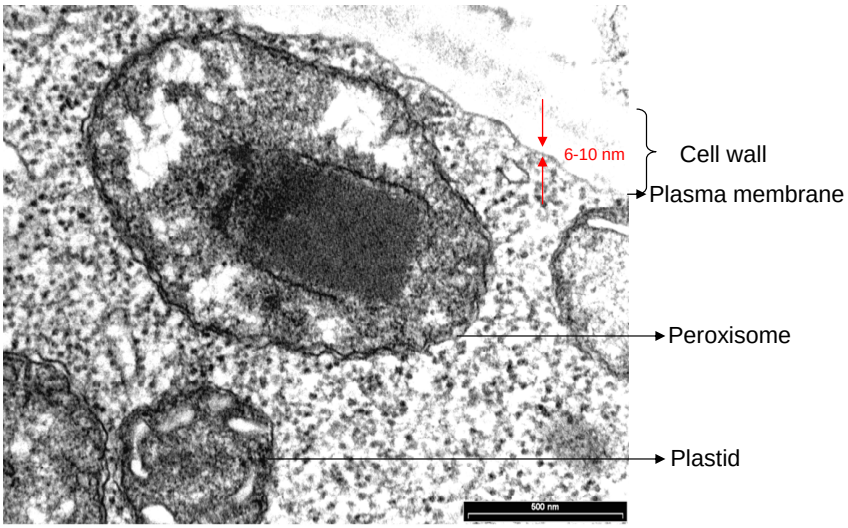


Figure 5.1: *Electron microscopic image of a banana meristem cell which demonstrates the small size of the plasma membrane compared to other membrane structures (made by B. Helliot).*

A major obstacle in PM proteomics is the low abundance of plasma membranes compared to other cellular membranes (see figure 5.1). Enrichment of the original sample in plasma membrane proteins is thus of utmost importance.

Some common techniques for plasma membrane isolation are described in chapter 2 and by Cordwell and Thingholm (2009). A widely applied technique in plant PM studies is aqueous two phase partitioning, first described by Larsson and Widell (Widell et al., 1982). As explained in chapter 2, subsection 2.2.1.1 on page 35, membrane partitioning is based on the different physicochemical properties of plasma- and endomembranes, resulting in a different affinity for the used polymer solutions (i.e., PEG and dextran). Sometimes only specific PM proteins such as aquaporins (Van Wilder et al., 2008) or PM H^+-ATPases (Osses and Godoy, 2006) are of interest. Other research projects, such as this PhD study, focus on the characterization of the complete PM proteome.

Although classical 2-DE has been applied for protein analysis (e.g., Malakshah et al. (2007); Zhang et al. (2008); Chen et al. (2007c)), the nature of PM proteins (low abundant, hydrophobic, sometimes basic and with high molecular mass) makes this technique less suitable. Therefore, peptide-based approaches are the methods of choice in PM proteome studies. Consequently, most PM studies have been applied on model plants such as *Arabidopsis* (Marmagne et al., 2007), rice (Natera et al., 2008) and poplar (Nilsson et al., 2010). In this chapter three different strategies (i.e., one protein-based and two peptide-based strategies) are combined to characterize plasma membrane proteins from banana leaves. In a first strategy, proteins from the PM fraction are solubilized in 4% SDS and separated by 1-D SDS-PAGE. In the peptide-based approaches the combination SCX-RP or the combination RP-RP is used for peptide separation. We focus on the problems that are associated with protein identification in a peptide-based approach in poorly sequenced plants and propose an identification strategy. For protein characterization we preferred to find the closest *Arabidopsis* homolog of the identified proteins to make use of the tools that have been developed for this model plant such as the plant proteome database which has already been useful in the previous chapters. We give an overview of the functional categories of the identified PM proteins to demonstrate the wide functional diversity that exists among PM proteins. Finally, we discuss the integral PM proteins that have been identified.

5.2 Materials and methods

5.2.1 Two phase partitioning

Banana leaves (20g, greenhouse plants, one year old Cachaco cultivar, ITC0643) were crushed and homogenized in 75 mL ice cold homogenization buffer, containing 100 mM Tris-HCl (pH 8.3), 5mM EDTA.Na_2, 85 mM KCl, 2% DTT, 0.33 M sucrose and complete EDTA-free protease inhibitor cocktail (Roche) using a cold kitchen coffee blender. Sample heating was prevented by applying short blending strokes. The homogenate was filtered through 4 layers of miracloth filter (Merck (Calbiochem), Darmstadt, Germany) and cell debris and nuclei were removed by centrifugation at 6500 *g* for 15 min at 4 ℃. Membrane vesicles were prepared through ultracentrifugation at 150,000 *g* for 1 h (4 ℃).

They were resuspended in 10 mL of 0.33 M sucrose, 0.1 M EDTA, 1% DTT, protease inhibitor cocktail and 5 mM potassium phosphate (Applichem, Darmstadt, Germany) buffer (pH 7.8). Plasma membranes were separated from the other membranes by aqueous two-phase partitioning as described by Larsson and Widell with slight modifications (Widell et al., 1982). Membrane vesicles (9 g) were added to phase systems containing 6.4% (w/w) dextran T500 (Pharmacosmos, Holbaek, Denmark), 6.4% (w/w) PEG 3350, 11% (w/w) sucrose, 6.67 mM potassium-phosphate (pH 7.8) and 6.67 mM KCl (Acros, Geel, Belgium), with a final weight of 36 g. Phase separation was enhanced by centrifugation at 1800 *g* for 5 min (4 ℃). The upper phases were three times re-extracted (figure 5.2). The final upper phase was diluted two times in resuspension buffer before ultracentrifugation at 100,000 *g* at 4 ℃ for 1h. Soluble contaminants were removed by washing the obtained pellets with 7 mL Brij58 buffer (150 mM KCl, 1 mM EDTA, 0.01% Brij58, 25 mM sodium acetate pH 4.0) by shaking during 30 min on ice. After a new centrifugation round (200,000 *g*, 45 min, 4 ℃), pellets were resuspended in the appropriate sample buffer and stored at -80 ℃.

The proteins in the PM fraction were identified using three different approaches which are discussed below. Of each approach two technical replicates were performed.

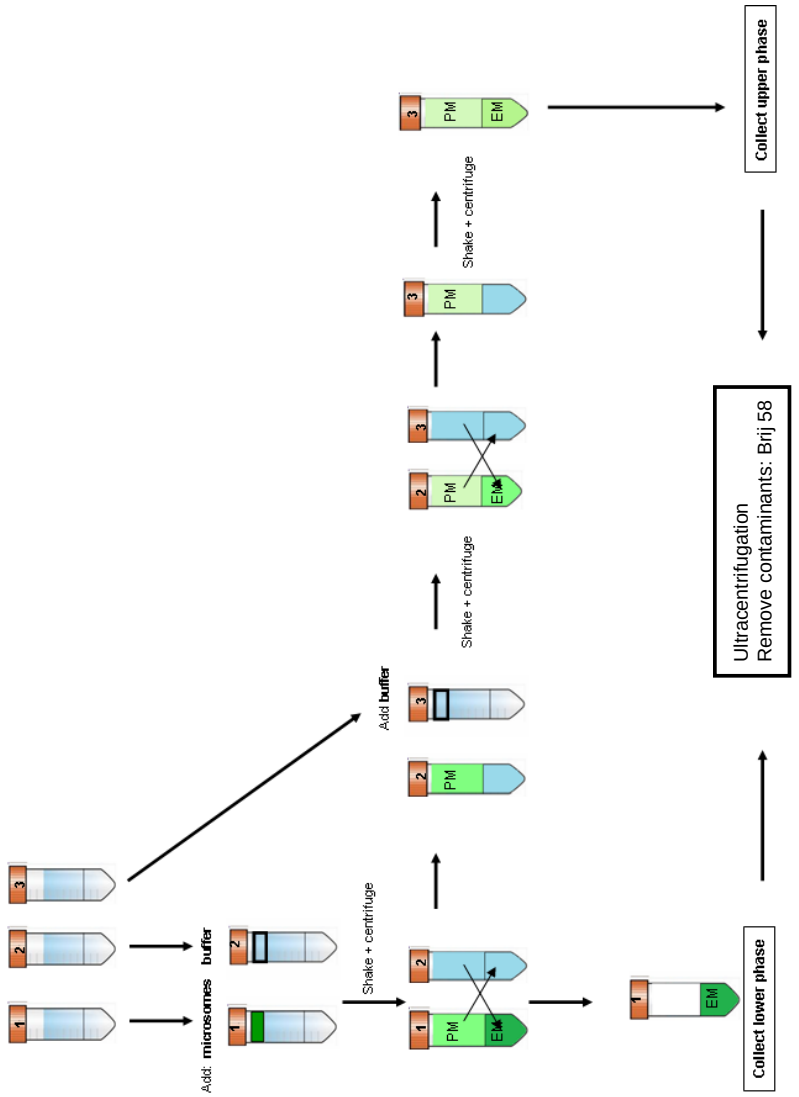


Figure 5.2: *The two phase partitioning protocol that was applied for the isolation of PM fractions from banana leaves.*

5.2.1.1 The protein-based approach

Proteins from the PM fraction were solubilized in 500 µL sample buffer containing 4% SDS, 1% DTT and 100 mM Tris-HCl pH 6.8. Samples were vortexed for 30 min at room temperature and protein concentration was determined using the micro-Bradford protocol as described by Zuo and Lundahl (2000). Before loading, samples were alkylated using 20 µM iodoacteamide, heated at 37 ℃ for 30 min and shortly centrifuged. Twenty µg of proteins were added to loading buffer yielding a final buffer concentration of 3% SDS, 75 mM Tris-HCl (pH 6.8), 15% glycerol, 3.5 M urea, 1% DTT and 0.05% bromophenol blue and loaded on a manually poured 12.5% acrylamide gel with a 4% stacking gel (18 × 24 cm, 1 mm thick). Gels were run overnight at 2W/gel at 12 ℃. For all separations the Laemmli protocol (Laemmli, 1970) was used. Gels were stained with G-250 Colloidal Coomassie (Neuhoff et al., 1988) and gel images were captured with labscan 5 software (GE Healthcare). After Coomassie blue staining and image analysis, stained bands were manually picked. In-gel digestion with trypsin and analysis of the tryptic peptides by MALDI TOF-TOF was performed at the "Centre de Recherche Public Gabriel Lipmann" in Luxembourg in the same way as described in chapter 3. For protein identification the NCBI *Viridiplantae* as well as the *Musa* database were searched. Protein identification was considered significant when protein score exceeded the MOWSE score and at least one peptide showed a significant ion score. A decoy database search was performed to estimate the number of false positive identifications.

5.2.1.2 A peptide-based approach using SCX-RP (HPLC) for peptide separation

Proteins from the PM fraction were solubilized in 1 mL 500 mM ammonium bicarbonate pH 8.0. After vortexing, samples were ten-fold diluted in the same buffer and centrifuged for 1 h at 100,000 *g* (4 ℃). The resulting pellet was washed with 5 mL 50 mM ammonium bicarbonate pH 8.0, vortexed (3 times 30 s), diluted (2 ×) in the same solution and plasma membranes were pelleted again by ultracentrifugation (100,000 *g*, 1h, 4 ℃). The pellet with the PM proteins was resolubilized in 400 µL 50 mM ammonium bicarbonate pH 8.0 and quantified using the micro-Bradford protocol. Samples were stored at - 80℃.

After thawing, methanol-assisted digestion was performed as described by Mitra and colleagues (Mitra et al., 2007). Briefly, methanol was added to the sample to yield a final ratio of 60:40 (MeOH: 50 mM ammoniumbicarbonate (pH 8.0)). After reduction with DTT (10 µM; 45 min at 60 ℃) and alkylation with iodoacetamide (20 µM, 90 min, dark, room temperature), trypsin was added in a ratio 1:20 (trypsin:protein). Digestion was performed overnight at 37 ℃. The methanol concentration was lowered to below 10% (v/v) in a Speedvac and a 10% volume equivalent of chloroform was added. The sample was thoroughly vortexed and phase separation was achieved after centrifugation at 16,000 *g* for 2 min. The upper aqueous layer was removed and vacuum dried. Peptides were stored at -20 ℃. Peptide separation and mass spectrometric analyses were performed at the Interfacultary Center for Proteomics and Metabolomics (Prometa) at K.U.Leuven. Peptides were resuspended in 10 µL of 5% ACN in water complemented with 0.1% formic acid (FA) and separated by 2-D LC MS/MS using an Ultimate 3000 system in nano-LC set-up (Dionex, US) coupled to a Q-TOF hybrid quadrupole time-of-flight mass spectrometer (Micro-Qtof, Bruker, Germany). Five µL sample was injected onto a 500 µm × 15 mm Bio-SCX column (LC Packings). The strong cation-exchange column was in-line with a reversed phase C18 precolumn (µ-guard column MGU-30 C18, LC Packings). Sample loading was done at a flow rate of 30 µL/min with water containing 2% ACN and 0.1% formic acid. Loading took 10 min. Peptides were eluted from the SCX column using ammonium acetate in different concentration steps. After adding 20 µL of the first ammonium acetate concentration, eluted peptides were immediately trapped on a pre-column. The SCX column was subsequently put off-line. The pre-column was rinsed for 5 min and switched in-line with the capillary column, a Pepmap C18, (3µm), 75µm × 150 mm nano column (Dionex). Peptides were separated from the latter column using a linear gradient from 95% solvent A, 5% solvent B to 5% A, 45% B in 40 min (solvent A: water, ACN, FA; 94.9, 5, 0.1 (% v,v,v); solvent B: water, ACN, FA (19.9, 80, 0.1 (% v,v,v)). The flow rate was set at 200 nL/min. The second fraction of peptides was eluted from the SCX column by injection of 20 µL of the next concentration ammonium acetate solution. The eluted peptides were again concentrated and desalted on the C18 precolumn prior to the MS/MS analysis. This cycle was performed with successive concentrations of 10, 80, 160, 240, 320, 480, 640 and 1280 mM ammonium acetate. This LC system was connected in series with the electrospray interface of a Q-TOF device (Thermo

Fisher Scientific). The column eluate was directed through a stainless-steel emitter (Proteon, Denmark). The needle voltage was set at 1800V. Nitrogen was used as nebulizing gas. Parent ions were automatically selected for further fragmentation by the software (compass 1.3, Bruker, Germany). Selection was based on signal intensity and charge state (double was preferred). Argon was used as collision gas; the collision energy was set at 25-40 eV depending on the mass and charge state of the selected ion. The detection window in the survey scan was set from m/z 400 to 2000. Fragmentation spectra were acquired from m/z 50 to 2000. All MS and MS/MS spectra were automatically processed using the FlexAnalysis software (Bruker, Daltonics) to generate peak lists in mgf format. Peak lists were submitted to a Mascot search against the *Musa* EST and NCBI *Viridiplantae* databases. A mass tolerance of 0.2 and 0.1 Da was allowed at the MS and MS/MS level respectively. Carbamidomethylation of cystein was set as fixed and oxidation of methionin as variable modification. For protein identification, only those proteins identified based on at least one significantly identified peptide were taken into account. Significance was determined by the Mascot MOWSE score. The FDR after a decoy search using the automatic search function of Mascot was estimated 0 at the MS/MS level.

5.2.1.3 A peptide-based approach using RP-RP (UPLC)

This section was executed at the Plant Research International Unit of the Wageningen University (Netherlands) by Dr. Sebastien Carpentier in cooperation with Dr. Twan America and Dr. Jan Cordewener. The pellet of the PM fraction was solubilized in 500 µL buffer containing 4% SDS, 1% DTT and 100 mM Tris-HCl. One hundred µL sample was mixed with 400 µL of 8 M urea in 0.1 M Tris/HCl (pH 8.5) (UA) and added to a Microcon YM-30 filter (Millipore, Billerica, MA, USA). The filter device was subsequently centrifuged at 14,000 *g* for 15 min. The filter was washed with 200 µL of the urea buffer UA and centrifuged again. Samples were incubated with 50 mM DTT for 15 min at room temperature and centrifuged at 14,000 *g* for 15 min. Subsequently, 100 µL of 0.05 M iodoacetamide in urea buffer was added. After 30 min, filter devices were centrifuged (14,000 *g*, 15 min). Two hundred µL of a buffer containing 8 M urea in 0.1 M Tris/HCl (pH 8.0) (i.e., UB) was added and centrifuged at 14,000 *g* for 15 min. This step was repeated twice. After these three washes Lys-C (in UB buffer) was added in an enzyme:protein ratio of 1:50. Proteins were mixed

with the enzyme and incubated overnight at room temperature. The next day, 120 µL of 40 mM ammonium bicarbonate with trypsin (enzyme:protein ratio of 1:100) was added and peptides were mixed with the enzyme at room temperature. Samples were incubated in a wet chamber at 37 ℃ for 4 hours. Filter units were centrifuged at 14,000 *g* for 20 min. After centrifugation, 50 µL of 50 mM NaCl was added and filter devices were centrifuged at 14,000 *g* for 20 min. Trifluoroacetic acid (0.5 %) was added to acidify and samples were desalted using solid phase extraction. Columns (Supelco Inc, Bellefonte, PA, USA) were washed with 1 mL 95 % ACN and equilibrated with 1 mL 2 % ACN, 0.1 % TFA. Subsequently, the tryptic digest was added to the columns. After a wash with 1 mL 2 % ACN, 0.1 % TFA, peptides were eluted with 1 mL 84 % ACN, 0.1 % TFA. Solvents were evaporated using a speedvac and peptides were dissolved in 40 µL 0.1 % ammonium formate (AF). Two dimensional LC-MS/MS experiments were performed using the 2-D nanoAcquity UPLC system online coupled to a Sybapt HDMS Q-TOF MS instrument (Waters, Milford, MA, USA). Four µL sample was injected on a first RP (bridge C18, 5 µm) column (300 µm × 50 mm, Waters). Elution from this column was stepwise under high pH and ultrahigh pressure at 2 µL/min. Samples were eluted in fraction of 12, 15, 18, 20, 25, 35 and 65% ACN in 20 mM ammoniumformate (pH 10). These fractions were optimized to ensure an equal distribution of peptides over the different fractions. Eluates were trapped on a C18 (Symmetry 5 µm) trap column (180 µm × 20 mm). Peptides were eluted in an excess of 0.1% FA in water at a flow rate of 20 µL/min to reach a ten fold dilution before loading on the analytical column (75 µm × 250 mm, BEH 130 C18 (1.7 µm)). Peptides were eluted from this column at 0.2 µL/min using 0.1% formic acid in water as eluent A and 0.1% formic acid in ACN as eluent B. The separation was carried out using 5% B for 1 min, 10% B for 2 min, 10-40% B over 62 min and 40-85% B over 9 min. After 6 min of rinsing with 85% B and a linear gradient back to 5% B over 2 min the column was re-equilibrated at initial conditions. The gradient slope was linear for 15, 18, 20 and 25% ACN, more concave for the 12% ACN fractions and more convex for the fractions 35 and 65% ACN. The analytical column temperature was maintained at 35℃ by a built-in column heater. Mass spectrometric analyses were performed in positive mode using ESI with a NanoLockSpray source. Eluates were immediately sprayed into a Q-TOF device (Waters). As lock mass, [Glu^1]fibrinopeptide B (1 pmol/µL) was delivered from a syringe pump (Harvard Apparatus, USA) to the reference

sprayer of the NanoLockSpray source at a flow rate of 0.2 µL/min. The lock mass channel was sampled every 30 s. For MS/MS, the three most intensive multiply charged ions eluting from the column were selected for fragmentation. The eluting peptide ions were detected in the MS survey scan (0.6 s) from m/z of 300 to 1400. A dynamic exclusion window was set at 60 s.

Obtained peak lists were searched against the *Musa* specific database using Proteinlynx Global Server (PLGS 2.3, Waters). Proteins identified with at least one peptide with a ladder score above 50 were considered significant. Unassigned spectra were used for *de novo* sequencing using the Proteinlynx software. These sequences, in combination with the peptides identified during the *Musa* search, were used for an MS homology[1] search. The number of allowed amino acid substitutions was determined as "the length of the peptide/5". Protein identifications were considered significant when protein scores exceeded 40.

A comparison of the three approaches is given in table 5.1.

[1] freely available at the website of the University of California (`http://www.ucsf.edu/`)

Table 5.1: *Comparison of three different separation approaches for PM proteomics used in this chapter. fr.=fractions*

	Protein-based	**SCX-RP**	**RP-RP**
Solubilization	4% SDS	AmBic: methanol (40:60)	4% SDS, replaced by urea using FASP
Digestion	Trypsin	Trypsin in presence of methanol	Lys-C (8 M ureum) Trypsin (2 M ureum)
Removal of lipids/interfering compounds	gel	chloroform	SPE
Separation	SDS PAGE	SCX-RP	RP-RP
Number of fractions	stained bands excised	SCX: stepwise AmBic(7 fr. + 1 wash) RP gradient; 40 min; 0-40% ACN	RP1: pH 11, stepwise ACN (6 fr. + 1 wash) RP2: pH 2.6, gradient 10-40-85% ACN
Chromatography	NA	HPLC	UPLC
Mass spectrometry	MALDI/TOF-TOF (ABI)	ESI/Q-TOF (Bruker)	ESI/Q-TOF (Waters)
Number of precursor ions fragmented	7	4	3
Time for fragmentation	NA	5 sec	5 sec
Database search	*Musa* NCBI *Viridiplantae*	*Musa* NCBI *Viridiplantae*	*Musa*
Cut off	MOWSE	MOWSE	ladder score
De novo sequencing	no	no	yes

5.2.2 Data analysis and informatics

The workflow that we applied for the identification and characterization of PM proteins in banana is demonstrated on figure 5.3. Peptides that were assigned to proteins in one of the three approaches were combined in one list and used for an MS Homology[2] search. Peptides assigned to keratin or trypsin were not taken into account for further analyses. The "length of peptide/5" rule was applied to calculate the number of allowed amino acid substitutions. In a first round peptides were searched against the Swiss-Prot green plants database. Not assigned peptides were searched against the NCBI *Viridiplantae* database in a second round. Results of both searches were combined. Peptides were ranked according to protein and peptide scores. Peptide identifications were estimated significant when protein scores exceeded 40. Peptides were filtered for redundancy. Protein-peptide associations were visualized using Cytoscape[3]. By combining a visualization of the results of the MS Homology search and the *Musa* searches, protein redundancy was estimated, wrongly associated peptides were recognized and removed and some proteins that were classified as unknown could be annotated. As such, a list of identified non-redundant proteins was created. From these proteins the *Arabidopsis* homolog was determined through a Blast search against *Arabidopsis*. This search was performed to retrieve information from the Plant Proteome database regarding the number of TMH, the GRAVY score, M_r and pI and functional MapMann annotation. The *Arabidopsis* gene identifiers (Agi) also allowed using GoMiner[4] (Zeeberg et al., 2003) to detect all proteins with a GO annotation for the cellular component plasma membrane. To characterize the proteins of which no *Arabidopsis* homolog could be detected, the different tools of the Expasy server were applied. ProtParam was used for the determination of GRAVY score, M_r and pI, TMHMM for the identification of transmembrane regions and TargetP to predict the protein location.

[2] `http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=mshomology`
[3] Freely available at `http://www.cytoscape.org/download.php`
[4] `http://discover.nci.nih.gov/gominer/`

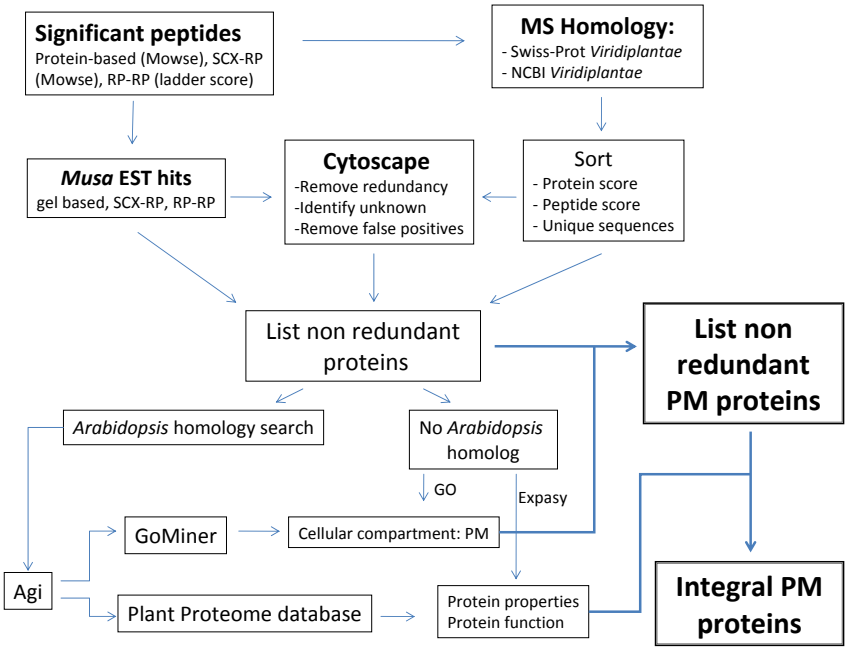


Figure 5.3: *The workflow followed for creating a list of non-redundant banana PM proteins identified by three different approaches.*

5.3 Results and discussion

5.3.1 Two phase partitioning

Plasma membranes constitute only 1.5-6% of the total proteome (Nilsson et al., 2010). This is too low to distinguish many PM proteins in a total protein pool. An initial enrichment in plasma membranes is thus of utmost importance in PM proteomics. Since plant cells, in contrast to animal cells, are protected by a rigid cell wall, plant protocols for PM purification generally consist of a combination of two methods; differential centrifugation and phase partitioning. We optimized both methods for use on banana tissue. In the differential centrifugation step, cell walls, unbroken cells and cell nuclei are eliminated. Generally also a part of chloroplasts and mitochondria is removed by applying a rather high centrifugation speed, i.e. 12,000 *g*. After removal of these heavy components, the remaining membranes are pelleted by ultracentrifugation.

We preferred to minimize the loss of plasma membranes in the first centrifugation step and maximize the recovery after the ultracentrifugation step and used a combination of 6500 *g* for the first centrifugation and 100,000 *g* for the ultracentrifugation step (not shown). In the subsequent phase partitioning step, PMs are separated from the bulk of membranes. In general, phase partitioning is based on the separation of cell components in two phases that are formed by mixing two structurally distinct water-soluble polymers above a certain concentration, i.e., the critical concentration. Each phase is enriched in one of the two polymers. The most widely used polymers in aqueous two phase partitioning for PM isolation are polyethylene glycol (PEG) and dextran (Johansson and Walter, 2000; Schindler and Nothwang, 2006). Both polymers are cheap, require only moderate concentrations, separate rapidly, have moderately low viscosities and can be easily buffered. Close to the critical concentration of both polymers, membranes, regardless of their subcellular origin, tend to partition into the top phase or at the interphase. By increasing the polymer concentrations, the difference in composition of the two phases becomes larger which results in a separation of plasma membranes from the other membranes. Plasma membranes show the highest affinity for the hydrophobic PEG-enriched top phase while chloroplasts and mitochondria, and in a lesser extent the endoplasmatic reticulum, lysosomes and Golgi vesicles, preferentially reside in the dextran rich lower phase (Widell et al., 1982). During the whole process, temperature should be kept constant because temperature fluctuations influence the separation process.

Plasma membrane isolation using this phase system is not complete after one phase separation. Even in the optimal case, the partitioning results in a ratio of 70% PM in the upper phase and 30% in the lower phase (Schindler and Nothwang, 2006). To obtain a purer PM fraction, a countercurrent distribution is generally performed. This involves a series of subsequent phase separations in which the upper phase is transferred to a fresh lower phase and the lower phase of the first system is re-extracted with a fresh upper phase. Schindler and Nothwang (2006) calculated that after three subsequent phase separations a PM fraction of sufficient purity is obtained. We preferred not to re-extract the lower phase since it also brings new endomembranes in the PM enriched fraction. The applied protocol is shown in figure 5.2.

A possible reason why plasma membranes also partition to the lower phase is the asymmetrical composition of this membrane, i.e., the protein and lipid composition of the apoplastic side differs from that of the cytoplasmic side (Tjellstrom et al., 2009). During initial cell homogenization PM vesicles are formed of which most have the apoplastic side on the outside (right-side-out, RSO). However, some of them have an inside-out orientation (ISO). These vesicles have a different polarity and a lower net charge which results in a lower affinity for the PEG upper phase as demonstrated by Larsson et al. (1994). The amount of RSO and ISO vesicles depends on the homogenization method used and the composition of the homogenization medium, but generally, 70-80 % of the vesicles are RSO (Johansson et al., 1995).

Since the most interesting proteins are located at the inside of the PM, methods were developed to turn the vesicles ISO. The most popular one is a wash with 0.05% Brij 58. This detergent permeates the PM at the apoplastic half of the lipid bilayer. Once incorporated, the detergent molecules force a change in the curvature of this side of the membrane which makes the RSO vesicles open. They reseal again as ISO vesicles. The detergent does not incorporate at the cytoplasmic side so originally ISO remain intact, yielding 100% ISO vesicles. The orientation is retained after removal of the detergent. The BRIJ wash was originally developed to study PM ATPase activity. We applied it because it has another important advantage. During a Brij wash soluble contaminants that are trapped inside the vesicle are released and can be removed after centrifugation. Johannson and his colleagues demonstrate that a large part of the small and large subunit of Rubisco are removed in this step (Johansson et al., 1995).

For the optimization of the two phase partitioning of banana leaves, the concentrations of PEG and dextran resulting in the best PM separation after three separation cycli were determined. Concentrations of both polymers were decreased or increased with the same % (w/w) to obtain a difference in composition between the tested two-phase systems. Because of the presence of chlorophyll, pureness of the PM fraction could be estimated by following the depletion of chlorophyll from the upper phase. As shown on figure 5.4, the 6.4% resulted in the highest depletion. These observations were confirmed by Western blot (antibodies against PM ATPase, not shown).

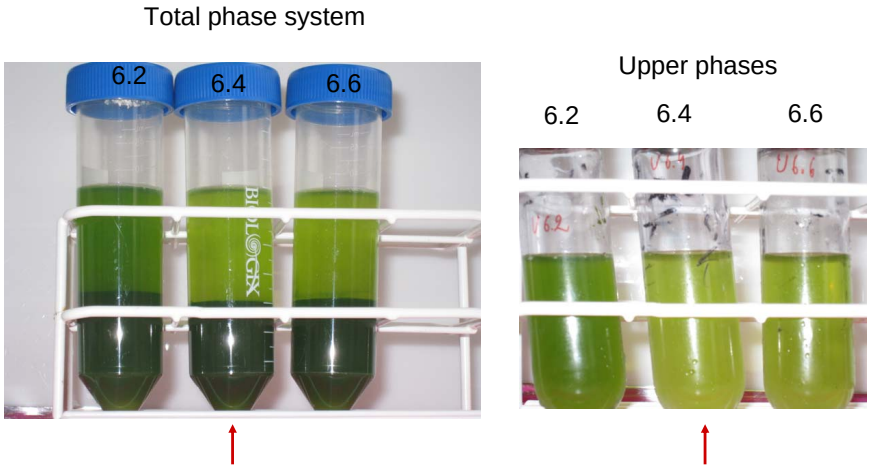


Figure 5.4: *Determination of the concentrations dextran T500 and PEG3350 yielding the highest degree of depletion of chlorophyll from the upper phase when applying two-phase partitioning. Left: total phase systems. Right: upper phases. The 6.4% (w/w) yields the clearest upper fraction (arrow).*

Also the band pattern of the proteins present in the upper and lower fraction demonstrates a difference between both fractions (figure 5.5)

Before peptide separation was performed, it was important to evaluate whether PEG was removed from the sample since this polymer could block the chromatographic columns and would interfere in the mass spectrum. The absence of PEG peaks in the mass spectrum after MALDI-TOF/TOF analysis of the complete PM sample confirmed the removal of the polymer (not shown).

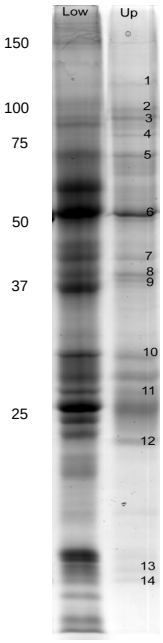


Figure 5.5: *The comparison of the protein band pattern in the lower (L) and upper (U) fraction after two phase partitioning. The numbers on the right lane indicate the bands with protein identification (see supplementary table all PM proteins). On the left, masses of the molecular weight standard (in kDa) are indicated.*

5.3.2 The protein identification strategy

For the characterization of the PM proteome of banana leaves, the results from three different strategies were combined (supplementary table all PM proteins). From the results of the previous chapters it was clear that when using a protein-based approach only a limited number of proteins is identified. Therefore, it was combined with two peptide-based approaches. In all three strategies two technical replicates were analyzed.

5.3.2.1 *De novo* sequencing, running technical replicates and the use of complementary techniques increases the number of identified peptides

It was not possible to make a real comparison between the three approaches because the protocols differed in too many factors such as the solubilization conditions and the accuracy of the mass spectrometer. However, it was clear that only a small number of peptides (i.e., 12) was identified in all three approaches (figure 5.6B). Moreover, in each of the three approaches some peptides and proteins were identified that were not identified by another approach (figure 5.6A), illustrating the value of using different approaches for protein identification.

Most peptides and proteins were identified in the RP-RP (UPLC) approach. An important reason is the high resolution obtained in this approach by performing chromatography under ultra high pressure (UPLC). This minimized the overlap of peptides over the different fractions and ensured that mass spectra were of high quality. The former allowed the identification of a higher number of peptides since the chance that the same peptide was identified in different fractions was much smaller compared to the SCX-RP approach. The latter (high quality spectra) made the application of *de novo* sequencing possible. These sequences are subsequently matched using BLAST against the sequences of known tryptic peptides present in the sequence databases. This allows the identification of spectra for which the exact peptide sequence is not present in the searched sequence databases.

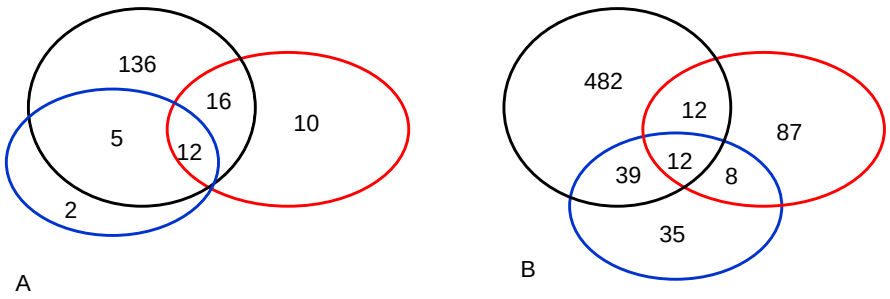


Figure 5.6: *The comparison of the proteins (A) and peptides (B) identified in the three approaches. Black=RP-RP; Red= SCX-RP, Blue= gel-based.*

It greatly improved the number of identified peptides. In the first RP-RP run, 74% of the peptides were identified through *de novo* sequencing, in the second run 60%. It is thus obvious that investing time in good mass spectrometric analysis, allowing *de novo* sequencing, greatly improves the number of identified peptides from poorly sequenced species.

Another important observation was that not only the three different approaches complemented each other, but that also in the two technical replicates different sets of peptides were identified. In the RP-RP approach where most peptides were identified, approximately only one third of the peptides assigned to *Musa* ID's were identified in both replicates (not shown). This observation highlights the importance of running technical replicates. Only in the protein-based approach little difference was observed between the two technical replicates. This is explained by the fact that the identification of a peptide depends on its chance to be fragmented. Since in the protein-based approach the number of peptides per well (of the MALDI plate) is relatively small, the chance a peptide is selected for fragmentation is higher compared to the peptide-based approach. During peptide fragmentation no peptide selection occurs. The time needed for fragmentation depends on the type of mass spectrometer. The shorter this time, the more peptides will be analyzed in the same time. This explains why in two different runs of the same sample different peptides were identified.

5.3.2.2 Pitfalls in a peptide-based approach on poorly sequenced plants

The purpose of most proteomic experiments is not the identification of peptides, but the identification of the proteins present in the sample before digestion. Thus, the peptide sequences need to be assigned to their corresponding protein. The strategy we followed for protein identification is shown in figure 5.3. First, the peptides identified in the three approaches were combined in one list which was used for an MS Homology search against the Swiss-Prot and NCBI *Viridiplantae* databases. Subsequently, significant protein identifications were estimated. A main challenge was protein redundancy, meaning that one peptide sequence was present in more than one entry in the protein sequence database. This is illustrated in figure 5.7. In the applied peptide-based approaches one peptide sequence was searched against a pool of peptide

sequences derived from all proteins which are entered in the Swiss-Prot or NCBI *Viridiplantae* databases. In these databases the same protein description is entered several times because it occurs in several species (i.e., protein homologs). Since banana is genetically divergent from most sequenced plants, we allowed amino acid substitutions (see materials and methods). A drawback is that there will be a large amount of proteins to which this one peptide hits (see figure 5.7B). We used the hit with the least amino acid substitutions. In a protein-based approach this problem is smaller since several different peptides of the same protein all have to match to one protein which decreases the number of possible hits (figure 5.7, left side).

It was thus important to handle the protein identification with the greatest care. By looking at the proteins that were identified in the protein-based approach and the protein score these proteins achieved after an MS Homology search in the peptide-based approach, we could have an indication of which peptide and protein scores were significant. In addition, we could rely on, although limited, species specific sequence information. In some cases, this allowed us to have an estimation of the closest protein homolog but it was impossible to correctly identify the exact protein isoform. Therefore we preferred to select one representative protein among possible isoforms and homologs and listed only protein descriptions (e.g., PIP1 instead of PIP1.1).

However, it is clear that sometimes several isoforms of the same protein were identified (see supplementary table all PM proteins). The identification of different variants of the same peptide, of different *Musa* ID's and of different Agi within the same protein description, is indicative for the presence of several isoforms of this protein in our sample.

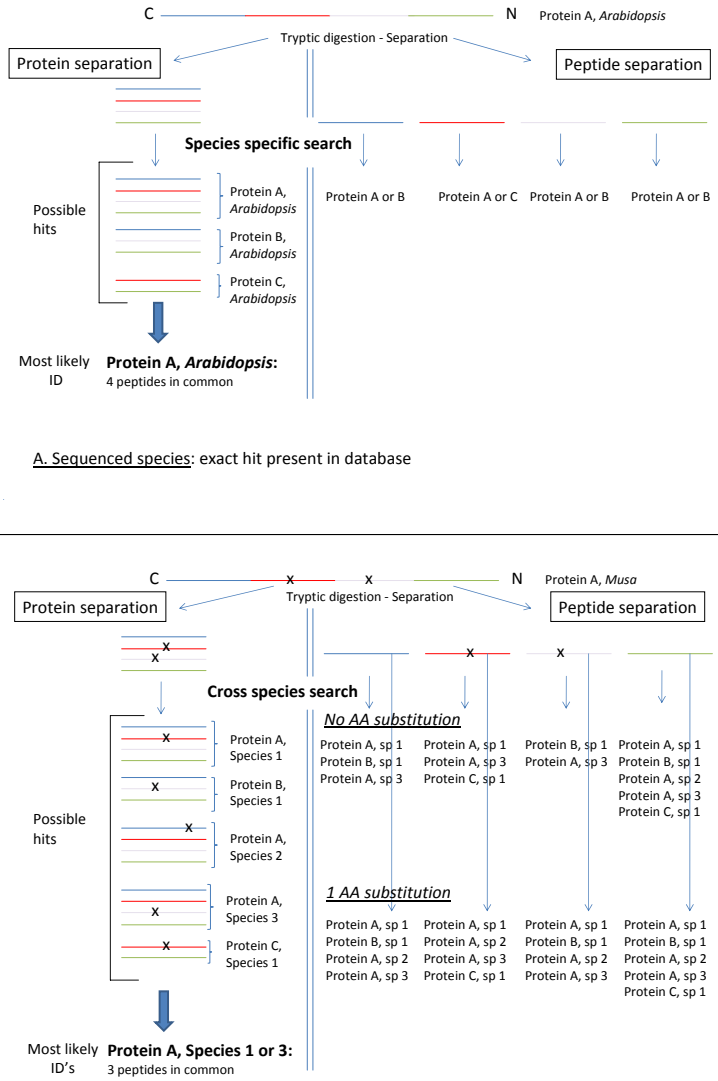


Figure 5.7: *Upper part. Protein identification in a model species. In a protein-based approach (left) peptides belonging to the same protein are still "connected" during mass spectrometric analysis which facilitates protein identification. The protein to which the most peptides are assigned receives the highest protein score and is the most likely ID. In the peptide-based approach (right) the connectivity is lost which makes protein identification harder. Lower part. Protein identification in poorly sequenced species is dependent on cross species searches. This increases the number of hits obtained. In general it is only possible to give a protein description (i.e., protein A) since the exact ID (protein A, Musa) is not present in the database. The x indicates an AA substitution in comparison to the protein of the model species.*

5.3.2.3 The use of Cytoscape to facilitate protein identification and visualize protein redundancy in banana

Another problem encountered in the peptide-based approach is the occurrence of false positive identifications, i.e., the assignment of peptides to proteins to which they do not belong. This mainly occurs in the cross-species approach were amino acid substitutions are allowed. A visualization program such as Cytoscape can facilitate the detection of these peptides, as proposed by T. America (personal communication). Using this program, peptides (circles on figure 5.8) that are assigned to a specific protein (squares) are connected with it by a line. The color of the line is indicative for the peptide score. As such all peptides assigned to one protein description form a cluster (figure 5.8 A). In case one peptide is assigned to two protein descriptions, it can easily be detected as a line that connects two of these clusters. This is illustrated in figure 5.8 B. Results from the *Musa* and MS Homology searches were simultaneously visualized in one Cytoscape view. The peptide with sequence QAIVGDLR was assigned to both the 14-3-3 (MS Homology search) and hypersensitive induced protein (HIR, *Musa* specific search) description. As it was assigned to the HIR protein with more confidence (green line) it was removed from the 14-3-3 protein (red line) list.

The use of Cytoscape also allows to visualize protein redundancy. Moreover, *Musa* specific peptides can be assigned to proteins identified based on homology of other peptides of the same protein. The ALCADHINVHLVTVPSAK peptide for example was not assigned to an *Arabidopsis* protein but since it was assigned to *Musa* ID's that also contained a peptide of the 12S subunit of the 40S ribosomal protein, it could be assigned to the 12S subunit (figure 5.9).

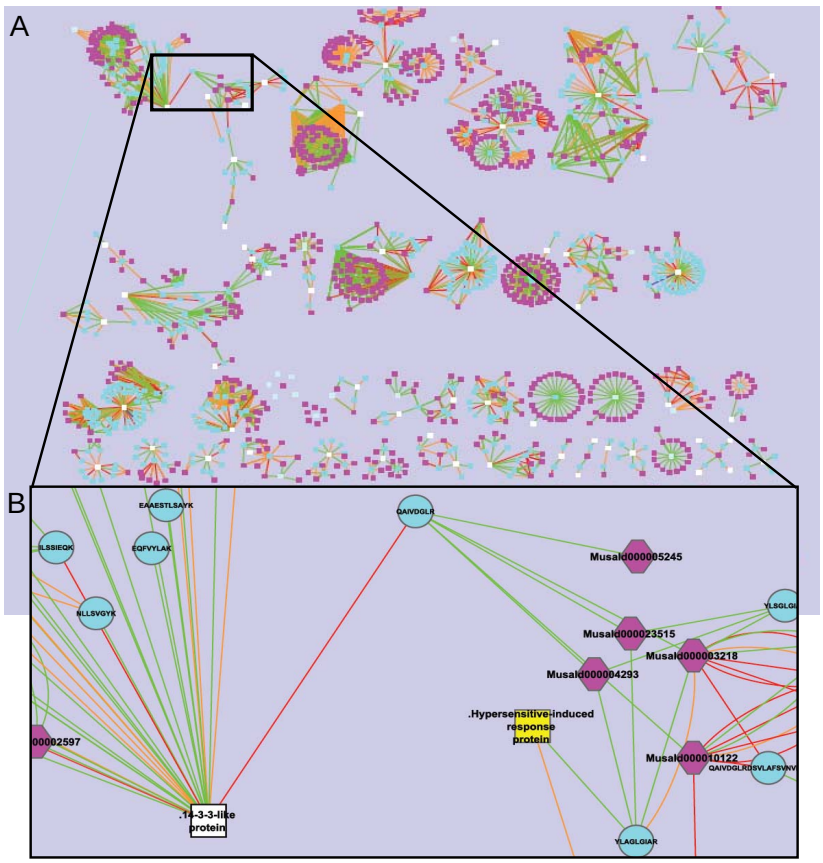


Figure 5.8: *A. Cytoscape is used to visualize the interaction between peptides and the proteins to which they are assigned. Squares represent protein descriptions, circles peptide sequences and lines the assignment of a peptide to a specific protein description. B. Illustration of a wrongly assigned peptide. Colors of the lines indicate ladder scores; red= $\leq$50, orange= 50-65, green= $\geq$65.*

Another interesting example is the ubuiquitin/27a ribosomal protein. This 40S ribosomal protein is synthesized as a C-terminal extension protein of ubiquitin. In Cytoscape this is nicely visualized by the interaction of the CGLTYVYQK protein with the 40S ribosomal protein as well as the ubiquitin protein description (not shown). In addition, Cytoscape also allowed to give a protein description to unknown *Musa* proteins.

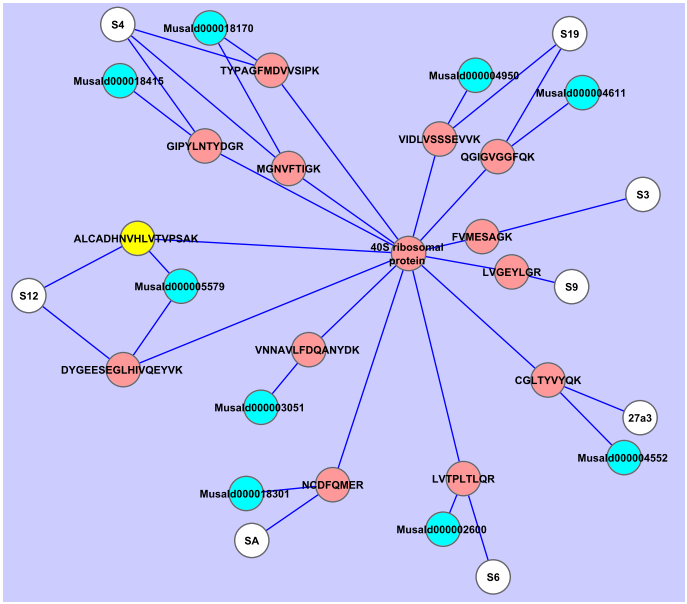


Figure 5.9: *The use of Cytoscape allows to assign Musa specific peptides to known protein homologs. Red circles represent the peptides that were identified and assigend to the different subunits of the 40S subunit (red circle in the middle). By the combined visualization of the Musa hits (blue circles) and the protein descriptions obtained from the Viridiplantae homology searches (white circles), the yellow peptide could be assigned to the 12S subunit.*

5.3.2.4 The use of *Arabidopsis* homologs to characterize banana proteins

Based on the Cytoscape analyses, a list with non-redundant protein descriptions was created (table all PM proteins, supplementary materials). To characterize the identified proteins we preferred to define the closest *Arabidopsis* homolog of the identified proteins. This was done by performing an MS Homology and Blastp[5] search against the Swiss-Prot *Arabidopsis* database. We preferred this approach since *Arabidopsis* is the best annotated plant. It allowed us to use the tools that have been developed to list and search characteristics of *Arabidopsis* proteins such as the Plant Proteome database and GoMiner.
We want to stress that using this approach we can only give indications about possible functions and other protein properties of the *Musa* proteins. The real *Musa* characteristics might differ from the ones listed in table all PM proteins.

[5] `http://blast.ncbi.nlm.nih.gov/Blast.cgi`

Proteins of which no homolog was found in *Arabidopsis* were characterized by using the different characterization programs as explained in the materials and methods section.

Since plasma membrane proteins, in contrast to chloroplast and mitochondrial proteins, can not be recognized based on a sequence specific sequence, we used the GO (Gene Ontology) annotation for the cellular component plasma membrane as a criterion to define a protein as being PM associated. Some contaminants from other cellular fractions were identified as well. Most were chloroplast proteins. This indicates that the two-phase partitioning should be optimized further.

5.3.3 Plasma membrane proteins

Taken into account the different subunits of the ribosomal proteins and the three classes of receptor like kinases, in total 88 non-redundant proteins were associated with the plasma membrane (table GO PM proteins, supplementary materials). Proteins from a wide range of M_r and pI were detected. As already mentioned, we did not list the different protein isoforms because of lack of complete sequence information and because we were mainly interested in the functional distribution of the different protein descriptions (figure 5.10). For this analysis, the different subunits of the ribosomal proteins were not taken into account. Figure 5.10 demonstrates that PM proteins are involved in many physiological processes, most of which are associated with cell signaling or transport of molecules. This is in contrast to the previous chapters were almost all identified proteins had a function in the photosynthesis process.

It is worthwhile mentioning that several other proteins, such as the fructose-bisphosphate aldolase, cytochrome b5, calreticulin and carbonic anhydrase, have been reported to be PM associated as well. Therefore, we also mentioned whether the identified proteins are present in the list of proteins published by Ephritikhine et al. (2007) (table all PM proteins, column M, supplementary materials). These authors compared several of the most important PM proteome publications and created a table with proteins identified in these studies[6].

[6]This table is freely downloadable `http://www.grenoble.prabi.fr/`.

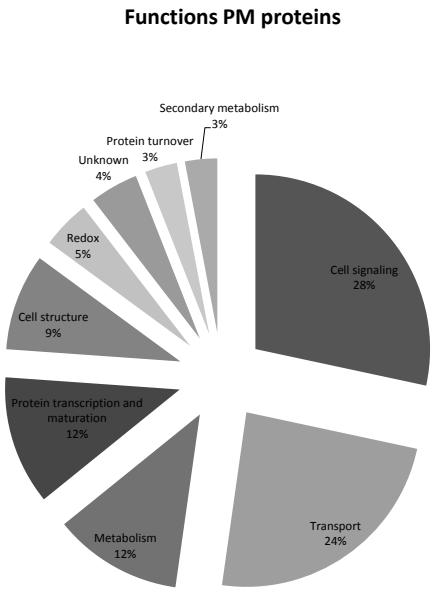


Figure 5.10: *The functional distribution of PM proteins, categorized based on the functional categories proposed by Ephritikhine et al. (2007).*

Since our main goal was to identify integral plasma membrane proteins, we further focused on this class of proteins (table 5.2).

5.3.4 Integral plasma membrane proteins

In total 20 of the identified plasma membrane proteins of table GO PM proteins (supplementary material) were predicted to contain at least one transmembrane domain. Compared to other publications, this number appears to be low, but we could not list the different isoforms of one protein. In table 5.2 we indicate the existence of isoforms by listing the number of different *Arabidopsis* gene identifiers (Agi) associated with one protein description and by listing the different variants of one peptide sequence that were identified. For example, we detected five different variants of one PIP1 peptide (figure 5.11). This shows that at least 5 different isoforms of the protein exist in the analyzed banana variety.

Table 5.2: *Integral membrane proteins in the PM fraction of banana leaves, listed according to their function. Proteins were identified as described in the materials and methods. The number of predicted TMH, the total number of peptides (Pept), the number of non redundant peptides (NR) and the number of NR above the significance treshold (> T) identified per protein, the possible isoforms based on the sequences of the identified peptides (Iso seq.) or the number of Arabidopsis gene identifiers (Iso Agi) per identified protein are shown.*

Function	**Protein description**	**TMH**	**Pept**	**NR**	**> T**	**Iso seq**	**Agi**
Transporters	Plasma membrane ATPase	10	42	28	20	3	7
	ABC transporter B family member	12	2	2	2	1	1
	Pyrophosphate-energized vac. membrane proton pump	16	8	5	5	2	1
	V-type proton ATPase						
	subunit A	0	8	8	7	1	1
	subunit B	0	1	1	1	1	1
	subunit E	0	2	2	2	1	1
	Polyol transporter	11	2	1	1	2	1
	Zinc transporter protein	8	1	1	1	1	1
	Aquaporin PIP1	6	28	9	9	8	6
	Aquaporin PIP2	6	15	6	6	3	4
	Delta tonoplast intrinsic protein	6	1	1	1	1	1
Receptors	Receptor like kinase (RLK):	1					
	a. Leucine rich repeat	1	9	9	8	1	7
	b. Serine/threonine	1	6	5	5	1	3
	c. *Catharanthus roseus*-like	1	16	12	10	3	6
	Probable receptor-like protein kinase	1	3	3	2	1	1
	Calcium-dependent protein kinase	1	6	6	5	1	4
	Membrane steroid-binding protein 2	1	1	1	1	1	1
Cell wall, cell elongation	Callose synthase	16	1	1	1	1	1
Membrane trafficking	Syntaxin	1	2	2	2	1	2
	Vesicle-associated membrane protein	1	2	2	1	1	1
	Putative lipid transfer protein	1	2	2	2	1	1
	Secretory carrier membrane protein	4	1	1	1	1	1
Other	Adenosylhomocysteinase (chaperone function)	1	1	1	1	1	1
	Senescence-associated protein	1	1	1	1	1	1
	Enolase	1	4	3	1	2	1

```
MusaId000021770     GLYENNGGGANVVAAGYTKGDGLGAEIVRTFILVYTVFSATDAKRSARDSHVPILAPLPI 223
MusaId000029688     GLYENNGGGANVVAAGYTKGDGLGAEIVGTFILVYTVFSATDAKRSARDSHVPILAPLP- 222
MusaId000018522     GLYENNGGGANVVAAGYTKGDGLGAEIVGTFILVYTVFSATDAKRSARDSHVPILAPLPI 225
MusaId000025870     GLYENNGGGANVVAAGYTKGDGLGAEIVGTFILVYTVFSATDAKRSARDSHVPILAPLPI 221
MusaId000025978     GLYENNGGGANVVAAGYTKGDGLGA---------------------------------- 204
MusaId000026470     GSMRQRWWG-NVVAAGYTKAMAWV------------------------------------ 202
MusaId000023103     GLYENNGGGANVVAAGYTKGDGLGAEIVGTFILVYTVFSATDAK---------------- 210
MusaId000020064     GLYENNGGGANVVAPGYTKGDGLGTEIVGTFILVYTVFSATD------------------ 205
MusaId000018472     GVYESNGGGANVVASGYSKGDGLGAEIVGTFILVYTVFSATDAKRNARDSHVPLLAPLPI 221
MusaId000025852     GVYQSNGGGANVVASGYSKGDGLGAEIVGTFILVYTVFSATDAKRNARDSHVPILAPLPI 230
MusaId000020789     GVYESNGGGANVVASGYSKGDGLGAEIVGTFILVYTVFSATDAKRNARDSHVPLLAPLPI 221
MusaId000030082     GVYESNGGGANVVASGYSKGDGLGAEIVGTFILVYTVFSAADAKRNARDSPVPLLAPLPI 221
MusaId000030133     GVYESNGGGANVVASGYSKGDGLGAEIVGTFILVYTVFSATDAKRNARDSHVPLLAPLPI 221
MusaId000030119     GVYESNGGGANVVASGYSKGDGLGAEIVGTFILVYTVFSATDASGMPG-------TPMCL 214
MusaId000022783     GLYESNGGGANVVAPGYTKGDGLGAEIVGTFILVYTVFSATD------------------ 211
MusaId000022982     GLYESNGGGANVVAPGYTKGDGLGAEIVGTFILVYTVFSATDAKRNARDSHVPILAPSPL 229
MusaId000025371     GLYESNGGGANVVAPGYTKGDGLGAEIVGTFILVYTVFSATDAKRNARDSHVPILAPLPI 229
MusaId000024327     GLYESNGGGANVVAPGY------------------------------------------ 184
MusaId000028568     GLYESNGGGANVVAPGYTKGDGLGAEIVGTFILVYTVFSATDAKRNARDSHVPILAPLPI 230
MusaId000025156     GLYESNGGGANVVAPGYTKGDGLGAEIVGTFILVYTVFSATDAKRNARDSHVPILAPLPI 230
MusaId000023043     GLYESNGGGANVVAPGYTKGDGLGAEIVGTFTLVYTVFSATDAKRNARDSHVPILAPSPL 198
MusaId000026989     GLYESNGGGANVVAPGYTKGDGLGAEIVGTFTLVYTVFSATDAKRNARDSHVPILAPSPL 222
MusaId000028187     GLYESNGGGANVVAPGYTKGDGLGAEIVGTFILVYTVFSATDAKRNARDSHVPILAPLPI 230
MusaId000023604     GVYESNGGGANAVASGYSKGDGLGAEIVGTFILVYTVFSATDAKRNARDSHVPLLAPLPI 212
MusaId000023806     GVYESNGGGANVVASGYSKGDGLGAEIVGTFILVYTVFSATDAKRNARDSHVPLLAPLPI 212
MusaId000027055     GLYENNGGGANVVAAGYTKGDGLGAEIVGTFILVYTVFSATDAKRSARDSHVPILAPLPI 169
MusaId000027318     GLYENNGGGANVVAAGYTKGDGLGAEIVGTFILVYTVFSATDAERSARDSHVPILAPLPI 174
MusaId000025425     GLYESNGGGANVVAPGYTKGDGLGAEIVGTFILVYTVFSATDAKRNARDSHVPILAPLPI 207
MusaId000019632     GLYESNGGGANVVAPGYTKGDGLGAEIVGTFILVYTVFSATDAKRNARDSHVPILAPLPI 211
MusaId000007814     GLYESNGGGLTSWLLATPRVDGLGAEIVGTFILVYTVFSATDAKRNARDSHVPILAPLPI 96
MusaId000022386     GLYESNGGGANVVAPGYTKGDGLGAEIVGTFILVYTVFSATDAKRNARDSHVPILAPLPI 160
MusaId000004063     GVYQSNGGGANVVASGYSKGDGLGAEIVGTFILVYTVFSATDAKRNARDSHVPILAPLPI 98
                    *  ...  * .    .
```

Figure 5.11: *Five different isoforms of the same peptide of PIP1 (underlined) were detected in our sample using the three described approaches, indicating that a high redundancy of this protein exists in banana.*

When looking at the peptides identified from the PM ATPase (figure 5.12), it becomes clear that only peptides from non transmembrane regions are identified. This implies that proteins with short extracellular domains without tryptic cleavage sites will not be identified using our approach .

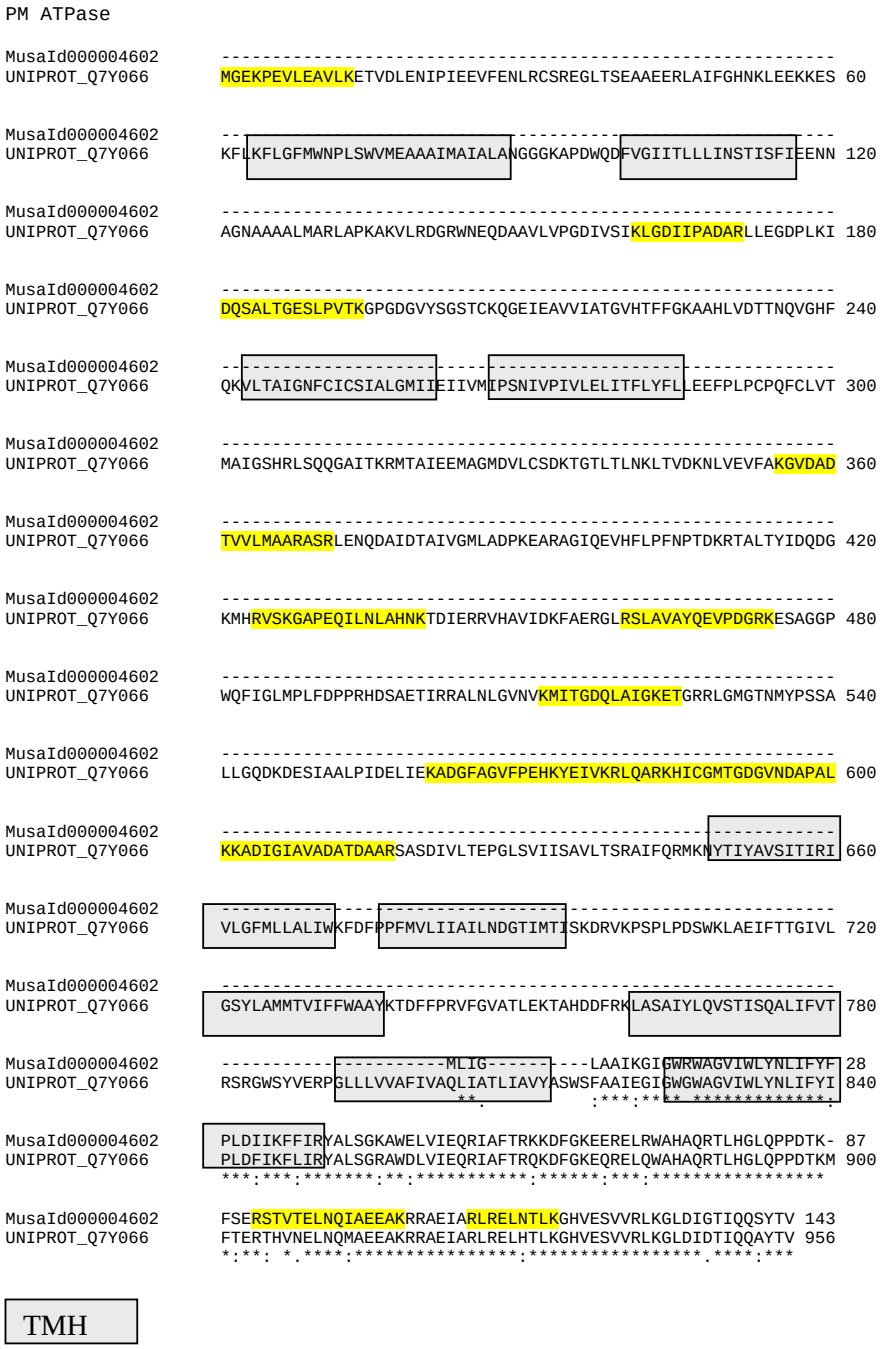


Figure 5.12: *The identified tryptic peptides of PM ATPase are indicated in yellow and the transmembrane domains (TMH) in gray boxes, demonstrating that the TMH do not contain tryptic cleavage sites.*

From table 5.2 it becomes clear that the most abundant PM proteins have a transport function.

A. Transporters One of the most abundant protein in our plasma membrane fractions is the plasma membrane H^+ ATPase. It was identified with 42 peptides, of which 28 were non-redundant (see table all PM proteins). This high redundancy can also be found in other plant species. It is likely that individual isoforms are tissue- and/or developmental-specific to assure pump activity in any cell at any given time of development. Indeed, this proton pumping ATPase exerts a crucial function (recently reviewed by Duby and Boutry (2009)). It couples the hydrolysis of ATP to proton transport out of the cell and generates as such an electrochemical proton gradient which drives other transport processes at the plasma membrane (Sondergaard et al., 2004). The inside-negative electrical potential across the plasma membrane also drives the K^+ import in the guard cells of stomata and regulates as such their opening. The activity of the PM ATPase is mainly regulated through phosphorylation events which are induced through the activity of 14-3-3 proteins (Oecking and Jaspert, 2009).

Other major PM proteins are the plasma membrane intrinsic proteins (PIPs). These proteins belong to the aquaporins or water channel family. In plants, this is a large family with 35 members in *Arabidopsis* (Johanson et al., 2001) and at least 33 in maize (Chaumont et al., 2001). The PIP subfamily can be further divided into PIP1 and PIP2 isoforms based on sequence homology. Also one tonoplast intrinsic protein (TIP) was identified. The latter protein has frequently been reported in PM studies (e.g., Marmagne et al. (2004); Alexandersson et al. (2004); Nilsson et al. (2010). Moreover, immunostaining demonstrated its localization at the PM (Ratajczak et al., 1999). Alexandersson et al. (2005) demonstrated that PIP transcripts and proteins play an important role in the responses on drought stress. A detailed study of these proteins and isoforms in banana could thus improve our understanding of drought stress responses.

The ATP binding cassette (ABC) transporters are pumps that actively transport a broad range of substrates including hormones, secondary metabolites, lipids, sterols and drugs across a membrane (nicely reviewed by Verrier et al. (2008)). These proteins are involved in many different processes such as plant-

pathogen interactions, detoxification and cutin accumulation at the plant surface (Theodoulou, 2000). Even the evolution of the seed size in tomato has been associated with an ABC transporter gene (Orsi and Tanksley, 2009).

Two other pumps that were identified in our PM fractions are usually associated with the vacuolar membrane, i.e., the vacuolar transporting pyrophosphatase and the V-type ATPase. Like the TIPs, also these pumps are frequently reported in proteomics studies of plasma membranes (Alexandersson et al., 2004; Marmagne et al., 2007) and could be localized to plant plasma membranes by immunostaining (Ratajczak et al., 1999). It was therefore suggested that the plasma membrane acts as a "temporary repository for tonoplast proteins en route to the vacuole" by Robinson et al. (1996).

Ion channels, such as the Zn transporter, are only needed in small amounts because of their very high transport capacities (Hacisalihoglu et al., 2001). Consequently, they are generally detected by a small number of peptides. We could only assign one peptide to a Zn transporter (table 5.2). Also the polyol transporter was detected with a small number of peptides. Its presence in PM fraction of leaves is explained by the need of carbohydrates for photosynthesis.

B. Receptors Another class of PM proteins is involved in receiving signals (table 5.2). The largest family of receptors in plants is composed of the receptor like kinases (RLKs), with over 600 members in *Arabidopsis* and over 1100 in rice (Shiu et al., 2004). Their abundance in plants is much higher compared to animal RLKs which is probably a plant-specific adaptation for extracellular signaling because plants are sessile. Receptor like kinases typically contain an N-terminal extracellular signal sequence, a transmembrane region and a C-terminal intracellular kinase domain for propagation of the signal (Shiu and Bleecker, 2001). They could play a role in the response of a plant on several types of stress such as wounding (Szczegielniak et al., 2005), drought or salt stress (Saijo et al., 2000) or cold stress (Martin and Busconi, 2001). The largest subfamily, the leucine-rich repeat (LRR) RLKs, have an extracellular domain with 1 to 32 LRRs which can interact with pathogen specific molecules or hormones (Zhang, 1998). The *Catharanthus roseus* like receptors (Schulze-Muth et al., 1996) form the second largest group. It is suggested that both groups of RLKs control cell growth. Calcium dependent protein kinases are a family of serine/threonine kinases that contain three functional domains: i.e., a

catalytic, autoinhibitory and calcium binding domain (Satterlee and Sussman, 1998).

Membrane steroid-binding proteins mediate the binding of steroid hormones and are characterized by a highly conserved single membrane spanning domain in the N-terminal region. In plants, the best studied steroid molecules are the brassinosteroids. These hormones are involved in the regulation of multiple developmental processes such as cell elongation (Yang et al., 2005) and can provide some protection to plants during chilling and drought stress (Krishna, 2003).

C. Cell wall and cell elongation Since plasma membranes are in close contact with the cell wall, proteins that are directly involved in cell wall synthesis and cell elongation are identified in PM fractions. We identified callose synthase, a very large protein (225 kDa) that regulates the formation of callose. The enzyme forms a protein complex with tubulin (Aidemark et al., 2009) and is probably involved in the response to environmental stresses since it is known that callose synthesis is rapidly up-regulated upon diverse stress situations (Sivaguru et al., 2000).

D. Vesicle transport and membrane trafficking Another important class of proteins detected in our PM fractions are the proteins involved in vesicle trafficking. Syntaxins and vesicle-associated membrane proteins (VAMPS) belong to the SNARE (soluble NSF attachment receptor) family proteins. These proteins mediate membrane fusion and have a key role in endocytotic cycling of membranes, including the recycling of receptors and other plasma membrane proteins (Murphy et al., 2005). They are also involved in exocytosis (Battey et al., 1999), and play thus also a role in cell wall formation.
Also secretory carrier membrane proteins (SCAMPs) (Hubbard et al., 2000) and lipid transfer proteins (De Matteis et al., 2007) likely play a role in membrane trafficking or its regulation.

5.3.4.1 Soluble proteins

Also several soluble proteins, known to be associated with plasma membranes, were identified in our PM fractions (table all PM proteins in the supplementary material). As already mentioned, 14-3-3 proteins are important regulators

of the PM ATPase activity (Oecking and Jaspert, 2009). The identified ribosomal proteins were probably attached to the plasma membrane via the cytoskeleton during extraction. Small GTP binding proteins might play a role in signaling pathways leading to the delivery of new cell wall components to the plasma membrane. Profilins are known to regulate cytoskeleton dynamics (Witke, 2004) and play specific roles in cell elongation and cell division. The class of proteins involved in protein turnover is almost exclusively composed of soluble proteins. However, the presence of these proteins in the PM proteome is not unexpected since the apoptotic signaling pathway can be induced by many extracellular stimuli. Marmagne et al. (2007) demonstrate that many of the soluble proteins found in a PM proteome are peripheral proteins that transiently interact with the PM.

5.4 Conclusions

In this chapter we described the isolation and identification of proteins from plasma membrane fractions of banana leaves using three different approaches. We thereby focused on the problems that are associated with peptide and protein identification in a peptide-based approach and proposed a possible workflow for identification. This workflow was based on the following findings; (i) performing technical replicates and *de novo* sequencing greatly increases the number of peptides identified (ii) the visualization of peptides that have been assigned to a specific protein helps to remove wrongly assigned peptides and to create a list with non-redundant protein descriptions and (iii) the number of peptides identified depends on the resolution power and mass accuracy of the used mass spectrometer. The availability of species specific information was instrumental in the identification of protein isoforms and in the validation of the results. Using this strategy, we could successfully identify a relatively large number of membrane proteins in comparison with the approaches used in the previous chapters of this thesis. However, the main challenge remains low abundant proteins. Moreover, the reproducibility of the PM isolation and protein analysis needs more confirmation.

Chapter 6

General conclusions and perspectives for future work

6.1 Conclusions

In this dissertation some techniques for membrane proteomics were evaluated for application in banana, a poorly sequenced plant. Our main goal was to complement classical 2-DE studies and to characterize the membrane proteome of banana meristems.

In a first approach we used a mixture of chloroform and methanol to specifically extract hydrophobic proteins from total cellular lysates. This method was preferred since it was reported to be very selective which allows starting from total cellular lysates without making a prior selection of proteins. We concluded that (i) since only a limited number of proteins are soluble in a C/M mixture, C/M extraction should always be complemented with alternative enrichment techniques, (ii) C/M soluble proteins are probably lipid-associated instead of highly hydrophobic proteins but this hypothesis needs further confirmation and (iii) total cellular lysates contain too many abundant soluble contaminating proteins which renders them unsuitable as starting material for membrane proteome studies. Nevertheless, the method might be instrumental

in chloroplast studies since Rubisco was almost completely depleted after a C/M extraction. We propose to use it as a delipidation technique on purified membranes and to analyze both C/M soluble and insoluble fractions.

In the next chapter we evaluated blue native electrophoresis as a method to enrich samples in proteins belonging to a membrane protein complex and to visualize protein-protein interactions. Since in the previous chapter total cellular lysates proved to be unsuitable for membrane proteomics, we preferred total membrane fractions as starting material. Only a small number of integral membrane proteins was identified, probably because of the mild solubilization conditions used. Further optimization was thus required to find the optimal solubilization conditions and to increase the number of membrane proteins identified. Regarding the use of BNE for the study of protein-protein interactions we concluded that total membrane fractions are too complex to provide a clear visualization of the interactions.
However, we believe in the power of BNE to provide preliminary information about existing protein-protein interactions in case there is no previous knowledge about a possible bait protein. For future studies, it is recommended to (i) use isolated membranes or organelles as starting material (ii) increase the amount of starting material (iii) evaluate the use of alternative detergents and to (iv) use BN-LC for protein identification since in this approach protein losses associated with the transfer from the first to the second dimension are avoided.

Since from our study we could conclude that the best way for membrane proteomics is using a specific purified membrane as starting material, we focused in chapter 5 on the plasma membrane proteome. Proteins associated with this membrane are the first sensors of changes in the environment and generate an appropriate intracellular response. A study of the plasma membrane proteome is thus essential for a better understanding of the drought response in banana. In addition, from the previous chapters it was clear that the resolution of the used protein based techniques for membrane proteomics is limited. Therefore a gel-based technique in combination with two gel-free approaches was used. The number of identified proteins and identified integral membrane proteins was clearly higher in comparison with the previous results. Most proteins were identified in the approach that used SDS for protein solubilization, and two

RP columns for peptide separation under ultrahigh pressure. The main reason for this higher number of identifications is that the obtained high quality mass spectra allowed *de novo* sequencing. Many of the identified membrane proteins were involved in the response to environmental stresses and should be studied in more detail in the framework of the research on the response of banana plants to different environmental stresses. However, some improvements should still be made such as (i) the use of alternative enzymes for digestion as trypsin only digested non-transmembrane regions and (ii) the exclusion of peptides from abundant proteins to improve the chance of fragmentation of peptides from less abundant proteins. The described procedure will also be used for the identification of PM proteins of banana meristems. It will be the first time this proteome is studied.

In total, 56 non redundant proteins containing one or more transmembrane domains were identified when combining the results from the previous chapters (table 6.1). More than half of them (31) were identified in the last chapter.

Table 6.1: *Integral membrane proteins identified in the three chapters. The protein description, the number of transmembrane helices (determined by Aramemnon) and the method used (C/M = chloroform/methanol extraction; BN= blue native electrophoresis; PM= plasma membrane isolation) are listed.*

Protein description	**TMH**	**C/M**	**BN**	**PM**
LEAF				
Pyrophosphate-energized inorganic pyrophosphatase	16			x
Callose synthase	16			x
ABC transporter B family member	12			x
Photosystem II D1 protein	11	x		
Photosystem I P700 chlorophyll a apoprotein A2	11		x	
Polyol transporter	11			x
Plasma membrane H+ ATPase	10			x
Photosystem II D2 protein	9	x		
LIMR family protein Os06g0128200	9			x
Similar to early-responsive to dehydration protein-related	9			x
Zinc transporter protein	8			x
Aquaporin PIP1	6			x
Aquaporin PIP2	6			x
Photosystem II CP43 protein	6		x	x
Delta tonoplast intrinsic protein	6			x
Secretory carrier membrane protein	4			x
Cytochrome b6/f complex subunit IV	3	x		
Cytochrome b-559 alpha subunit	2	x		
Photosystem I reaction center subunit V	2	x		
Photosystem I reaction center subunit VI	2			x
Photosystem I reaction center subunitIII	2			x
Chlorophyll a-b binding protein type 2 member 2	2	x	x	
Chlorophyll a/b binding protein of LHCII type III	2	x		
Chlorophyll a-b binding protein CP24	2	x		x
Light-harvesting complex IIa protein	2	x		
Peroxidase 43	2			x

continued on next page

continued from previous page

Protein description	TMH	C/M	BN	PM
PSAL	2		x	x
RNA polymerase II C-terminal domain phosphatase-like 1	2			x
Chlorophyll a/b-binding protein type I	1	x		
Vesicle-associated membrane protein	1			x
Rieske FeS protein precursor (Cytochrome b6-f complex iron-sulfur subunit)	1	x		
Lhcb1*9	1	x		
Glycolate oxidase	1	x	x	
Syntaxin-132	1			x
Peroxisomal malate dehydrogenase	1	x		
Putative receptor protein kinase	1			x
Adenosylhomocysteinase	1			x
Membrane steroid-binding protein 2	1			x
Receptor like kinase (RLK)	1			x
Cysteine-rich receptor-like protein kinase 27	1			x
Cytochrome b5	1			x
Ferredoxin	1			x
Senescence-associated protein	1			x
Enolase	1			x
Putative lipid transfer protein	1			x
Calcium-dependent protein kinase	1			x
Transketolase	1		x	
MERISTEM				
Pyrophosphate-energized vacuolar membrane proton pump	15	x		
Probable aquaporin PIP2-6	5	x		
ADP,ATP carrier protein 1, mitochondrial	3	x		
Cytochrome c1-2, heme protein, mitochondrial	2		x	
Uncharacterized protein	1	x		
Cysteine proteinase inhibitor 6	1	x		
Uncharacterized protein	1	x		
Chromosome chr11 scaffold_13, whole genome shotgun sequence	1	x		
Uncharacterized protein	1	x		

Based on these observations we can make some general conclusions regarding membrane proteomics in poorly sequenced plants, being

(i) Sufficient time should be invested in the optimization of the isolation of the membrane of interest

(ii) The highest recovery of membrane proteins is obtained when a strong detergent such as SDS is used for protein solubilization. This detergent has to be removed before enzymatic digestion.

(iii) Protein separations using gel electrophoresis are limited in power. A major problem in this approach is that proteins or peptides need to be extracted from gel. Because of the limited resolution, more than one protein will be present in one stained band. Only those peptides that are easily extracted from the gel will have a high S/N ratio and will be identified.

(iv) Gel-free approaches have a higher resolution power compared to gel-based approaches in membrane proteomics and avoid the extraction from gel. Peptide separation needs to be optimized to ensure an equal distribution of peptides over the different fractions and to limit the number of shared peptides over the different fractions. Care should be taken during peptide and protein identification. A possible workflow is suggested in chapter 5.

(v) The use of complementary approaches and/or technical replicates increases the number of identified proteins

(vi) The use of trypsin as a digesting enzyme only identifies peptides from non transmembrane regions. To improve the recovery of peptides from transmembrane regions alternative digesting methods could be used in combination with trypsin. However, the use of these enzymes will not solve the problems associated with solubilization and separation of the peptides from hydrophobic regions such as the retainment of hydrophobic peptides on the LC columns. Moreover, these peptides will generally not have the high ionization capacity of tryptic peptides.

(vii) Application of *de novo* sequencing greatly improves the number of identified peptides and proteins.

(viii) Since all purified membrane fractions will also contain some contaminants from other compartments it is difficult to predict the exact location of a protein. Location prediction programs, GO annotations and similarity with homologous proteins will give an indication. However certainty only comes from location studies were stained probes are used to visualize the location of a protein.

(ix) For poorly sequenced plants only indications about a protein function, the number of TMH and its location can be obtained. The actual properties can differ from those of the closest homolog.

In summary, we suggest the following workflow for membrane proteomics in banana

(i) Use a purified membrane or organelle as starting material and SDS for protein solubilization

(ii) Remove SDS by one of the techniques described in chapter 2, section 2.3.1.1

(iii) For the characterization of a specific membrane proteome:

   a. Use a sufficient amount of starting material

   b. Use a combination of a geLC and 2-D LC separation

   c. Ensure the highest resolution possible (e.g., the mass spectrometer of choice)

(iv) For quantitative studies :

   a. Use gel-free techniques

(v) For the study of protein-protein interactions

   a. Use BN-LC for protein identifications

   b. Use BNE for the visualization of protein-protein interactions

(vi) For protein identification

a. Perform a similarity search against the *Musa* specific database

b. Perform *de novo* sequencing on the unassigned mass spectra

c. Perform a homology search against the NCBI database (*Viridiplantae*) of the significantly identified peptides

d. Remove protein redundancy and false positives using Cytoscape

e. Find the closest *Arabidopsis* homolog

f. Use the tools developed for the characterization of *Arabidopsis* proteins to characterize the *Musa* proteins

g. Characterize the other proteins using the available tools

6.2 Future plans

In the previous chapters we did not focus on the quantitative aspect of membrane proteomics in banana because it requires that membrane purification and protein separation and identification are optimized. Moreover, protein quantification becomes troublesome when a series of subsequent purification steps are performed prior to the actual quantification step. In every step a possibility of significant and irreproducible protein/peptide losses exists. The more steps are performed before the actual quantification step, the less controllable the quantification becomes. In comparison with the soluble proteome, at least one additional step has to be performed to extract membrane proteins; i.e., the isolation of the membranes. In case one specific membrane is isolated, which is recommended, even more isolation steps are required. One strategy could be to add a label prior to all purification steps and to combine all samples during further analysis like in the SILAC approach. However, this method is only applicable to *in vitro* cultures and preferable to cell cultures. Therefore, most quantitative studies on membrane proteins use the iTRAQ labeling strategy or label-free quantification.

Quantitative studies of banana plasma membranes will be very challenging because we observed that the phase partitioning used to enrich PM's is not reproducible between banana cultivars. An AAA cultivar yielded a purer PM fraction compared to an ABB cultivar when exactly the same two-phase partitioning was applied. To cope with this problem, an internal standard could be used to calculate relative concentrations in each cultivar. This internal standard should be a protein of which the concentration does not differ between treatments nor cultivars. The concentration of the extracted proteins can subsequently be compared to this "house-keeping" protein. The ratios obtained in both cultivars will give an indication about differential proteins.

As such, optimization of quantitative membrane proteomics in poorly sequenced species becomes the challenge for future research. In case of banana, we recommend to focus this research on the PM proteome since several of the identified proteins had a role in the reaction to drought or other environmental stresses.

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Part III

Appendix

Appendix A

Additional tables and figures

This appendix contains additional tables and figures from the different chapters. Further supplementary material can be found online.

Tables from chapter 2

- Publications on membrane proteomics in plants
- Publications on the use of BN in plants

Table A.1: *Publications on membrane proteomics in plants. Listed are: the separation method; the digestion method; the plant species and organelle where the experiment was performed on; the reference of the corresponding paper and the % of membrane proteins identified. NA= not applicable.*

Separation	Digestion	Plant	Organelle	Author	Membr. prot. id.
Gel-free methods					
RP	NA	Barley, spinach, *Arabidopsis*	chloroplast, thylakoid	Huber et al. (2004)	NA
size exclusion + RP	trypsin or CnBr ... ...and intact proteins	NA	chloroplast, thylakoid	Whitelegge (2004)	NA
Mudpit	trypsin	*Arabidopsis*	PM	Alexandersson et al. (2004)	47% (114/238)
LOPIT	trypsin	*Arabidopsis* callus culture	fractionation of organelles	Dunkley et al. (2004)	NA
Mudpit	trypsin in Brij 58 ... ...or methanol	*Arabidopsis* seedlings	microsomes + PM	Mitra et al. (2007)	43.3 % with MeOH
Mudpit	Lys-C	Orange	total proteome	Katz et al. (2007)	NA
Mudpit	trypsin	*Arabidopsis* leaf	plastid and mitochondria	Lee et al. (2007)	22% (93/415)
RP	trypsin	*Arabidopsis*	PM	Nühse et al. (2007)	NA
Mudpit, in comb. with geLC	trypsin	Rice	PM	Natera et al. (2008)	53% (425/805)
Mudpit	trypsin	*Arabidopsis*	PM	Mitra et al. (2009)	NA
Mudpit	trypsin	Maize seedlings		Shen et al. (2009)	34% (25/73)
Mudpit and 2-DE	trypsin	Soybean	PM	Komatsu et al. (2009)	NA
RP	trypsin	Rice	DRM	Fujiwara et al. (2009)	52% (26/50)
RP	trypsin	Lily	pollen	Pertl et al. (2009)	NA
RP	trypsin	*Arabidopsis*	chloroplast, thylakoid	Laganowsky et al. (2009)	NA
Gel-free and gel-based methods					
gel filtration, anion exchange, ...SDS-PAGE	trypsin	*Arabidopsis*	vacuole, tonoplast	Szponarski et al. (2004)	60% (42/70)
SDS-PAGE, RP	trypsin	*Arabidopsis* cell susp.	PM	Marmagne et al. (2007)	30% (136/446)
SDS-PAGE, RP	trypsin, chymotrypsin, ...limited acid hydrolysis		chloroplast, thylakoid	Friso et al. (2004)	49% (76/154)
SDS-PAGE or ... ...phase partitioning , RP	trypsin	*Arabidopsis*	chloroplast, thylakoid	Peltier et al. (2004)	40% (97/242)
SDS-PAGE, RP	trypsin	*Arabidopsis*	chloroplast, envelope	Ferro et al. (2003)	92% (34/37)
SDS-PAGE, RP	trypsin	Cauliflower	vacuole, tonoplast	Schmidt et al. (2007)	32% (102/316)
SDS-PAGE, RP	trypsin	*Arabidopsis*	PM	Marmagne et al. (2004)	51% (53/120?)
SDS-PAGE, RP	trypsin	*Aradidopsis*	PM, lipid rafts	Morel et al. (2006)	78% (28/36)
SDS-PAGE (2DE and 1-D), RP	trypsin	*Arabidopsis*	PM, lipid rafts	Borner et al. (2005)	NA
SDS-PAGE, RP	trypsin	*Arabidopsis* cell susp.	mitochondria	Brugière et al. (2004)	68% (77/114)
2DE, RP	trypsin	*Arabidopsis* cell susp.	PM	Lanquar et al. (2007)	NA
BNE-RP	trypsin	*Dunaliella salina*	PM	Katz et al. (2007)	NA
RP, SDS-PAGE, RP	trypsin	Barley embryo's	PM	Hynek et al. (2009)	46% (28/61)
RP, SDS-PAGE, RP	trypsin	Barley seeds	PM	Hynek et al. (2006)	87% (41/47)
SDS-PAGE, RP ... ...in comb. with Mudpit	trypsin	*Oryza sativa*	PM	Natera et al. (2008)	53% (425/805)

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Separation	Digestion	Plant	Organelle	Author	Membr. prot. id.
SDS-PAGE, RP	trypsin	Poplar	PM	Nilsson et al. (2010)	22% (213/956)
off-line SCX-RP	trypsin	*Arabidopsis*	PM	Nelson et al. (2006)	51% (36/70)
SDS-PAGE (and 2)DE), RP	trypsin	Rice	PM	Hashimoto et al. (2009)	NA
SDS-PAGE,RP	trypsin	*Medicago*	PM	Lefebvre et al. (2007)	NA
SDS-PAGE, RP	trypsin	Rice	PM and vacuole	Whiteman et al. (2008)	41% (94/231)
Gel-based methods					
1-D SDS-PAGE	trypsin	Spinach	chloroplast, envelope	Ferro et al. (2002)	79% (42/53)
1-D SDS-PAGE	immunoblot	Broccoli seeds	root PM	Lopez-Perez et al. (2009)	NA
1-D SDS-PAGE	trypsin	Pea and maize	chloroplast	Bräutigam et al. (2008)	NA
16-BAC	NA	Pea	golgi vesicles	Wenzel et al. (2005)	NA
dSDS-PAGE	trypsin	*Arabidopsis* cell culture	peroxisomes	Eubel et al. (2008)	66% (25/38)
Classical 2-DE	trypsin	*Arabidopsis*	chloroplast	Fan et al. (2009)	20% (3/15)
Classical 2-DE	trypsin	Grape berry	PM	Zhang et al. (2008)	82% (51/62)
Classical 2-DE	trypsin	Rice	diff. subcellular compartments	Tanaka et al. (2004)	NA
Classical 2-DE	trypsin	Rice	root PM	Cheng et al. (2009)	NA
Classical 2-DE	trypsin	Rice	PM	Chen et al. (2007c)	NA
Mudpit and 2-DE	trypsin	Soybean	PM	Komatsu et al. (2009)	NA

Table A.2: *Publications on the use of BN in plants. The separation and digestion method, the used detergent (maltoside= n-dodecyl-b-D-maltoside), the plant and organelle where the experiment was performed on and a reference of the describing paper are listed. ED= Edman degradation.*

Separation	Digestion	Detergent	Plant	Organelle	Author
BNE/BNE-BNE	trypsin	digitonin, Triton X-100 ... ...and dodecylmatoside	*Arabidopsis*, potato, ...bean, barley	mitochondria	Eubel et al. (2003)
BNE	trypsin	digitonin and dodecylmatoside	*Arabidopsis*	mitochondria	Millar et al. (2004)
BNE	trypsin	dodecylmaltoside	*Arabidopsis*	mitochondria	Giege et al. (2003)
BNE	trypsin	digitonin	*Arabidopsis*	mit., outer membrane	Jänsch et al. (1996)
BNE	NA	dodecylmaltoside or digitonin	*Arabidopsis*, Polytomella	mit. and chloroplasts	Heinemeyer et al. (2009)
BNE/BNE-BNE	immunoblot	digitonin	Cucumber	mitochondria	Juszczuk and Rychter (2009)
BNE	ED, immunoblot	dodecylmaltoside	Potato	mitochondria	Jänsch et al. (1996)
BNE	trypsin	dodecylmaltoside	*Arabidopsis* and rice	mitochindria	Heazlewood et al. (2003b)
BNE	trypsin, lim. acid hydrolysis	dodecylmaltoside	Barley	photosynthetic app.	Ciambella et al. (2005)
BNE	immunoblot + unknown	dodecylmatoside	*Arabidopsis*	mit., outer membrane	Werhahn and Braun (2002)
BNE	trypsin	dodecylmaltoside	Spinach	PM	Kjell et al. (2004)
BNE	trypsin	dodecylmaltoside	Spinach	chloroplast	Fagioni et al. (2009)
BNE, BN-RP	trypsin	dodecylmaltoside	Maize	chloroplast	Majeran et al. (2008)
BNE	trypsin	dodecylmatoside	Sugar beet	chloroplast, thylakoid	Andaluz et al. (2006)
BNE	trypsin	dodecylmaltoside	Barley	chloroplast, thylakoid	Granvogl et al. (2006)

Tables from chapter 3

- Comparison of C/M extraction on whole cell lysates (our research) and isolated organelles (referring to the corresponding paper).

Table A.3: *Comparison of C/M extraction on whole cell lysates and isolated chloroplasts.*

Location	Agi	Name	Paper	Our research
CHLOROPLAST				
thylakoid; PS II	AtCg00280	psbC photosystem II 44 kDa protein	Friso 2004	dSDS
	AtCg00270	photosystem II D2 protein	Friso 2004	1-DE,dSDS
	At4g21280	oxygen-evolving enhancer protein 3-1	Friso 2004	1-DE
	At4g05180	oxygen-evolving enhancer protein 3-2	Friso 2004	1-DE, dSDS
	At1g29910	CAB1	Friso 2004	dSDS
	At1g29920	CAB2	Friso 2004	dSDS
	At3g27690.	Lhcb2	Ferro 2003, Friso 2004	1-DE, dSDS
	At5g54270	Lhcb3	Friso 2004	dSDS
	At5g01530	chlorophyll a-b binding protein CP29.1	Friso 2004	1-DE
	At3g08940	chlorophyll a-b binding protein CP29.2	Friso 2004	1-DE, dSDS
	At4g10340	chlorophyll a-b binding protein CP26	Friso 2004	1-DE, dSDS
	At1g15820	Lhcb6	Ferro 2203, Friso 2004	1-DE, dSDS
	At4g03280	cytochrome b6-f complex iron-sulfur subunit	Ferro 2003, Friso 2004	1-DE
thylakoid; PS I	AtCg00350	psaA photosystem I P700 chlorophyll a apoprotein A1	Friso 2004	
	AtCg00340	psaB photosystem I P700 chlorophyll a apoprotein A2	Friso 2004	1-DE, dSDS
	At1g31330	photosystem I reaction center subunit III	Friso 2004	1-DE, dSDS
	At4g12800	photosystem I subunit XI	Ferro 2003, Friso 2004	dSDS
	AT3g54890	Lhca1	Ferro 2003,Friso 2004	1-DE, dSDS
	At3g61470	Lhca2	Friso 2004	dSDS
	At1g61520	PSI type III chlorophyll a/b-binding protein	Ferro 2003, Friso 2004	1-DE, dSDS
	AT3g47470	Lhca4	Ferro, 2003 Friso 2004	1-DE, dSDS
	At3g11630	2-Cys peroxiredoxin BAS1	Friso 2004	dSDS
envelope	At4g38970.	probable fructose-bisphosphate aldolase 2	Ferro 2002	1-DE,dSDS
	Plastid gene	Rubisco large chain	Ferro 2002	1-DE
	At2g25080	phospholipid hydroperoxide glutathione peroxidase 1	Ferro 2003	1-DE
	At1g67090	Rubisco small chain 1A	Ferro 2003	1-DE

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Location	Agi	Name	Paper	Our research
MITOCHONDRION				
	At1g53240.	malate dehydrogenase 1, mitochondrial	Brugière 2004	1-DE, dSDS
PEROXISOME				
	At5g09660	peroxisomal malate dehydrogenase	Ferro 2003	1-DE, dSDS
		Proteins not found in C/M literature		
CHLOROPLAST				
thylakoid	AtCg00130	ATP synthase CF0 B subunit		dSDS
stroma	At2g37660	Y2766_ARATH		1-DE
MITOCHONDRION				
	At2g44350	citrate synthase 4, mitochondrial		1-DE
PEROXISOME				
	At2g13360	serine–glyoxylate aminotransferase		1-DE,dSDS
	At3g14415	(S)-2-hydroxy-acid oxidase, peroxisomal, putative		1-DE, dSDS
	At1g23310	putative alanine aminotransferase (ACC synthase)		1-DE
OTHER				
	At4g23600	tyrosine transaminase like protein (ACC synthase)		1-DE
	At5g02240	Uncharacterized protein At5g02240		1-DE

Other tables of chapter 3 can be found in the online supplementary material of this dissertation.

Figures from chapter 3

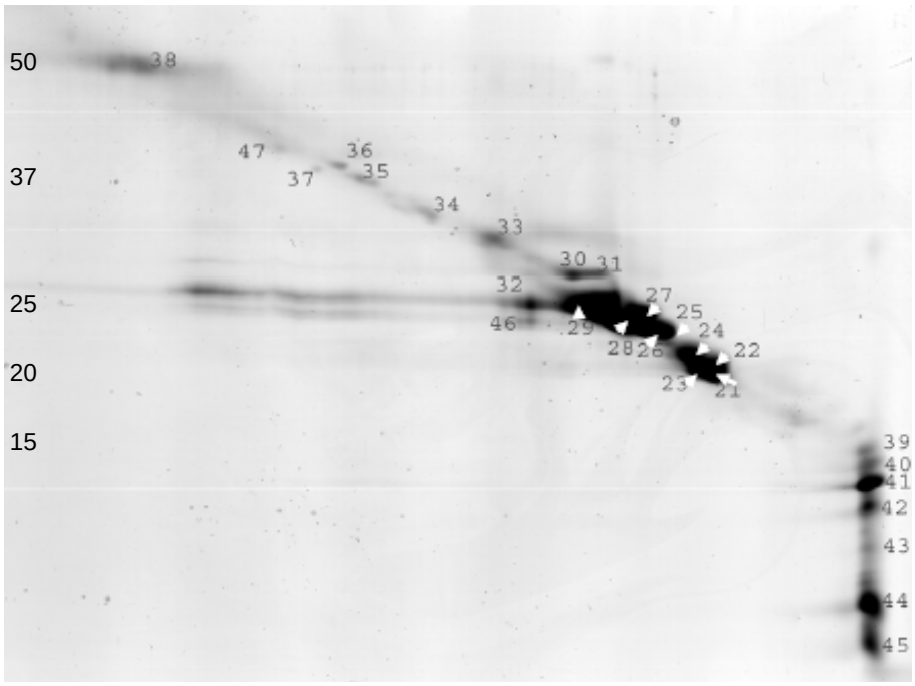

Figure A.1: *Double SDS gel of proteins from banana leaves soluble in 5/4 C/M. 40 µg of extract was loaded. Numbers indicate the number of the spot, corresponding to one or more identified proteins. Molecular masses of standard proteins are indicated on the left (kDa). Coomassie Brilliant blue stained gel.*

Tables from chapter 5

Tables all PM proteins and GO PM proteins can be found in the online supplementary materials.

Appendix B

List of publications

Articles in internationally reviewed scientific journals

- Carpentier, S.C., Vertommen, A., Swennen, R., Witters, E., Fortes, C., Souza, M.T. Jr., and Panis, B. 2010. Sugar-mediated acclimation: The importance of sucrose metabolism in meristems. *Journal of Proteome Research*, 9, 5038−5046.

- Vertommen, A., Panis, B., Swennen, R. and Carpentier, S.C. 2010. Evaluation of chloroform/methanol extraction to facilitate the study of membrane proteins of non-model plants. *Planta*, 231 (5), 1113−1125.

- Carpentier, S.C. , Panis, B., Vertommen, A., Swennen, R., Sergeant, K., Renaut, J., Laukens, K., Witters, E., Samyn, B. and Devreese, B., 2008. Proteome analysis of non-model plants: a challenging but powerful approach. *Mass spectrometry reviews*, 27 (4), 354−377.

Articles published in other scientific journals

- Vertommen, A., Panis, B., Swennen, R. and Carpentier, S., 2008. The study of hydrophobic proteins of Arabidopsis thaliana and banana, a model and non model crop. *Communications in Agricultural and Applied Biological Sciences*, 73 (1), 33−36.

- Vertommen, A., Carpentier, S., Remmerie, N., Witters, E., Swennen, R. and Panis, B., 2007. Towards the identification of protein complexes in banana (*Musa* spp.) mersitems. *Communications in Agricultural and Applied Biological Sciences*, 72 (1), 51−54.

Papers at international conferences and symposia; published in full in proceedings

- Vertommen, A., Carpentier, S., Remmerie, N., Witters, E., Swennen, R. and Panis, B. 2008. Study of hydrophobic proteins and protein complexes involved in cryopreservation of banana (*Musa* spp.) meristems. In: *CRYOPLANET - COST Action 871. Agrifood Research Working papers 153 Cryopreservation of crop species in Europe*, 19-20.

Meeting abstracts, presented at international conferences and symposia, published or not published in proceedings or journals

- Vertommen, A., Möller, A. L. B., Finnie, C., Svensson, B., Baggerman, G., Swennen, R., Panis, B. and Carpentier, S., 2009. The study of the *Musa* spp. plasma membrane proteome using a gel-based and gel-free approach. *COST FA0603 Plant proteomics in Europe WG2 & MC meeting.* Book of Abstracts 23. Nitra, Slovakia, 14-16 October 2009. Oral abstract.

- Carpentier, S. , Vertommen, A., Swennen, R., Sergeant, K., Renaut, J. and Panis, B., 2009. 2D DIGE, PCA/PLS and *de novo* identification, a powerful combination to explore crop biodiversity and to explain variety specific phenotypes. *COST FA0603 Plant proteomics in Europe WG2 & MC meeting.* Book of Abstracts 29. Nitra, Slovakia, 14-16 October 2009. Oral abstract.

- Carpentier, S. , Vertommen, A., Swennen, R. and Panis, B., 2009. Will proteomics contribute to a better understanding of cryopreservation survival? *1st International Symposium on Cryopreservation in Horticultural Species.* Leuven, Belgium, 5-8 April 2009.

- Vertommen, A., Möller, A. L. B., Finnie, C., Svensson, B.,Swennen, R., Panis, B. and Carpentier, S.C., 2009. Isolation of plasma membranes: next step towards a complete (banana) meristem proteome. Berlin, Germany, 29 March-2 April 2009. C063. Poster abstract

- Carpentier, S. , Henry, I., Vertommen, A., Swennen, R. and Panis, B. 2008. Will proteomics contribute to a more sustainable agriculture? Renaut J. , Hausman J.-F., Hoffmann L. (ed.). Proteomlux 2008. 25. *International Conference on Proteomics in Plants, Microorganisms and Environment.* Luxembourg, Grand Duchy of Luxembourg, 22-24 October 2008. Oral abstract

- Vertommen, A., Carpentier, S., Sergeant, K., Renaut, J., Swennen, R. and Panis, B., 2008. Chasing the right tools to study the hydrophobic proteome of *Musa* spp. meristems. Renaut J., Hausman J.-F., Hoffmann L. (ed.). Proteomlux 2008. 55 *International Conference on Proteomics in Plants, Microorganisms and Environment.* Luxembourg, Grand Duchy of Luxembourg, 22-24 October 2008. Oral abstract

- Vertommen, A., Carpentier S., Witters, E., Laukens, K., Swennen, R. and Panis, B., 2007. Towards the identification of hydrophobic proteins in banana (*Musa* spp.) meristems. *Organelle subproteomics and post-translational modifications* (joint meeting FNRS GCProt and BSBMB). ULB, Brussels, Belgium, 15 May 2007. Poster abstract .

- Vertommen, A., Carpentier, S., Witters, E., Laukens, K., Swennen, R. and Panis, B., 2007. Towards the identification of hydrophobic proteins in banana (*Musa* spp.) meristems. *Epigenetics and somaclonal variation in plants*. Espace Vincotte, Gembloux, Belgium, 23 November 2007. Oral abstract.

- Vertommen, A., Carpentier, S., Witters, E., Laukens,, K., Swennen R. and Panis, B., 2006. Study of the membrane proteome of banana (*Musa* spp.) meristems. *Symposium on Proteome Analysis in Systems Biology*. Antwerp, Belgium, 7-8 December 2006. Poster abstract. (second poster prize).