Chapter 11
The Pollen Membrane Proteome

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Abstract The male gametophyte (or pollen) is a highly specialized organ essential for sexual reproduction of higher plants. Their reduced complexity constitutes them as an ideal experimental system for analyses of biological processes maintaining tip growth. Rapid advances in proteomic technologies and a vast choice of metabolic labelling and label-free quantitation protocols as well as the availability of full genome sequences allow comprehensive analyses of various pollen proteomes. Pollen membrane proteome consists of integral and membrane-associated proteins involved in regulation of many cellular functions. In this chapter, novel insights into identification of membrane proteins by proteome analysis and how their dynamic subcellular localization contributes to the initiation of pollen grain germination and maintenance of tube growth are discussed.

Keywords Mass spectrometry • Membrane proteins • Protein-protein interactions • Proteomics

Abbreviations

2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
2D DIGE	Two-dimensional difference in gel electrophoresis
ACA	Autoinhibited-type Ca^{2+} ATPase
BiFC	Bimolecular fluorescence complementation
CaM	Calmodulin
COP	Coat protein complex
ECA	Endoplasmic reticulum-type Ca^{2+} ATPase
ER	Endoplasmic reticulum
ESI Q-TOF MS/MS	Electrospray ionization quadrupole time-of-flight tandem mass spectrometry
GFP	Green fluorescent protein

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G. Obermeyer, J. Feijó (eds.), *Pollen Tip Growth*,
DOI 10.1007/978-3-319-56645-0_11

IEF	Isoelectric focusing
LC-MS^n	Liquid chromatography coupled with multistage accurate mass spectrometry
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometer
mbSUS	Mating-based split-ubiquitin system
p*I*	Isoelectric point
PIP	Plasma membrane intrinsic proteins
PM	Plasma membrane
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
TGN	Trans-Golgi network
VDAC	Voltage-dependent anion channel

11.1 Introduction

Due to its simple morphology and highly specialized function, the growing pollen tube is a well-established model system to study tip growth mechanisms. In general, tip-focused growth of pollen tubes is regulated by a network of cellular processes and components that are mainly localized in the tube apex and subapex region. Many of these processes are related to membranes, e.g. membrane-associated cytoskeleton, a highly active membrane trafficking system for endo- and exocytosis, signal transduction pathways including membrane receptors, cytosolic Ca^{2+} concentration (Ca^{2+}_{cyt}) and pH (pH_{cyt}) as well as localized ion fluxes across the plasma membrane of pollen tubes and grains (Feijó et al. 1995, 2001; Holdaway-Clarke and Hepler 2003; Bibikova et al. 2004; Cheung and Wu 2008; Michard et al. 2009, 2017). Of special note, ion transporters in the plasma membrane are generating the typical current pattern of ion fluxes that possibly functions as a navigation system for the tube's growth rate and growth direction (Holdaway-Clarke and Hepler 2003; Michard et al. 2009, 2017). Fluxes of protons (H^+), potassium (K^+), chloride (Cl^-) and calcium ions (Ca^{2+}) across the plasma membrane contribute to cytosolic ion concentrations but also to the complex electrical current pattern along the pollen tube. Although electrophysiological techniques like the ion-sensitive vibrating probe or patch-clamp analysis give a detailed view of the ion current pattern around growing pollen tubes and characterize single ion transporters, they cannot identify the particular ion transporter protein or protein complex that is generating the current nor give a comprehensive view of all ion transporters involved in current pattern generation. In addition, signal transduction pathways are also involved in the regulatory network that contribute to activate or inactivate cellular processes including membrane receptors and components that modulate ion transporter activities like the cytosolic-free Ca^{2+} concentration, the cytosolic pH, protein phosphorylation and 14-3-3 proteins, G-proteins and phospholipids (Kost et al. 1999; Pertl et al. 2001; Potocky et al. 2003; Monteiro et al. 2005; Michard et al.

2008). All mentioned components were found to be essential for tip growth, and it is assumed that they also interact with each other forming a complex spatial-temporal regulatory network controlling the events necessary for pollen tube growth.

Despite the important role of the polar distribution of plasma membrane proteins, only a few plasma membrane proteins were localized in pollen tubes by immunolocalization or GFP-fusion proteins, including the PM H^+ ATPase (Obermeyer et al. 1992; Certal et al. 2008), a Ca^{2+} ATPase (Schiott et al. 2004), the SLAH3 anionic channel (Gutermuth et al. 2013), members of the cyclic nucleotide-gated channels (CNGC; Frietsch et al. 2007; Tunc-Ozdemir et al. 2013; Gao et al. 2016) and components of signal transduction pathways, e.g. calcium-dependent protein kinase (Myers et al. 2009; Gutermuth et al. 2013), CIPK/CBL pairs (Steinhorst et al. 2015) or inositol phosphate kinases (Ischebeck et al. 2008), among many others. However, an intrinsic property of a regulatory network is that all components are somehow connected and the network as a whole functions in tube growth. Therefore, we need to investigate as many network components as possible under the same experimental conditions to understand the dynamics of the pollen tube growth process entirely. A proteomic approach identifying the membrane proteins and membrane-associated signalling proteins may be a first step to understand the protein interaction network responsible for maintenance of pollen tube elongation.

11.2 Pollen Proteome

11.2.1 Basic Proteomic Approaches for Model and Non-model Organisms

Rapid advances in proteomic technologies are tightly associated with completion of genome sequences of model plants or relevant crop plants like *Arabidopsis* (Arabidopsis Genome Initiative 2000), rice (*Oryza sativa,* Yu et al. 2002; Goff et al. 2002), maize (*Zea mays,* Schnable et al. 2009), barrelclover (*Medicago truncatula,* Young et al. 2011), tobacco (*Nicotiana benthamiana,* Bombarely et al. 2012), tomato (*Solanum lycopersicon*, The Tomato Genome Consortium 2012) and poplar (*Populus trichocarpa,* Tuskan et al. 2006) as well as the availability of comprehensive public sequence databases. Functional composition analysis of the *Arabidopsis* pollen transcriptome revealed that the mRNAs presented in pollen mainly encode proteins involved in cell wall metabolism, vesicle transport, cytoskeleton and signalling (Becker et al. 2003; Honys and Twell 2003, 2004; Pina et al. 2005; Wang et al. 2008; Loraine et al. 2013) which reflects their functional specialization in initiation of pollen germination and maintenance of tube growth. Although the analysis of mRNA expression is useful for general studies, the presence or absence of a transcript does not necessarily reflect the expression of the encoded protein. Thus, in addition to transcriptome analyses, a detailed analysis at the protein level is an essential step towards the further identification of functional

components involved in pollen germination and tube growth. Proteomics, however, provide new insights on the mechanisms of pollen development and germination in higher plants (reviewed in Dai et al. 2007b), but so far, only few detailed proteome studies have been carried out on pollen considering the temporal dynamics of pollen tube growth process.

Recently, a comparative proteome analysis of tobacco pollen showed that pollen development can be divided into three phases. The early phase that ends with release of the microspores is characterized by the so-called sporophytic proteome, which is relatively static. During the intermediate phase, the sporophytic proteome is partially degraded, whereas the late phase presents a 'gametophytic proteome' that contains many proteins required for pollen tube growth and cell wall synthesis (Ischebeck et al. 2014). In addition, a combination of two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MALDI-TOF MS and ESI Q-TOF MS/MS) applied to mature rice pollen identified several new proteins involved in signal transduction, cell wall remodelling and metabolism, protein synthesis, assembly and degradation, as well as carbohydrate and energy metabolism (Dai et al. 2006). Also, new proteins involved in defence mechanisms, energy conversion, hormone metabolism and signalling, and membrane transport were identified in tomato pollen by proteome analysis (Sheoran et al. 2007). Quite recently, the pollen membrane proteome from two tomato cultivars was analysed to obtain a general overview on membrane function in pollen (Paul et al. 2016). In both cultivars, more than 150 proteins were assigned as putative membrane proteins, mainly involved in energy-related processes (Krebs cycle and glycolysis) that emphasize the protein content of mature pollen as energy reservoir for subsequent pollen germination and pollen tube growth. *Arabidopsis thaliana* is excellently suited for large-scale proteome analyses because of its small genome size and well-annotated gene databases. Of the approximately 29,000 genes in the *Arabidopsis* genome (Alonso et al. 2003), approximately 6600 are expressed in pollen, and only 10–20% of these genes are pollen specific (Holmes-Davies et al. 2005). Nearly half of the pollen-specific mRNAs were found to encode for proteins needed for building a cytoskeleton, for cell wall metabolism and for signal transduction that also supports the hypothesis that mature pollen grains contain mRNAs which then encode proteins for rapid germination and maintenance of pollen tube growth. In that context, most identified proteins are necessary for cell and cell wall structure (glycosyl hydrolase family protein, pectin methylesterase, pectin methylesterase inhibitor family protein, actin, actin-depolymerizing factors and tubulin), energy metabolism (ATPase synthase subunits, malate and isocitrate dehydrogenases and malate oxidoreductase) and signal transduction (calnexin 1, GTP-binding protein SAR1B, inositol 1,3,4-triphosphate 5/6 kinase, Holmes-Davies et al. 2005).

Proteome analyses of pollen were mainly performed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and by comparison between protein spot pattern of wild-type and mutant plants. 2D gel-based proteomics has valuable features, e.g. its reproducibility and robustness, which make it often an attractive choice when a proteomic strategy must be selected (Rabilloud 2014). Nevertheless, 2D-PAGE based on isoelectric focussing (IEF) is technically limited because it is

mainly restricted to soluble and/or high abundant proteins. In addition, 2D-PAGE is subject to restrictions, which include limited dynamic ranges and difficulties in handling hydrophobic proteins and in detecting proteins with extreme molecular weights and isoelectric point (p*I*) values. The use of this technique may account for the relative low number of identified membrane proteins in earlier pollen proteome studies (Dai et al. 2006, 2007b; Sheoran et al. 2006). Therefore, alternative approaches that are based on mass spectrometry techniques for identification of complex peptide or protein mixture and quantification methods have been developed. Mass spectrometry-based protein quantification represents an important enhancement to simple identification proteomics by enabling a comparison of protein expression levels between different samples or treatments. Comparative protein quantification by mass spectrometry typically employs stable isotope incorporation, such as isotope-coded affinity tag (ICAT), stable isotope labelling by amino acids in cell culture (SILAC), $^{15}N/^{14}N$ metabolic labelling and isobaric tags for relative and absolute quantification (iTRAQ). However, certain limitations, including additional sample processing steps, cost of labelling reagents, insufficient labelling, difficulty in analysis of low abundance peptides and limitation of sample number, are associated with the use of these labelling techniques (Zhang et al. 2013). As an alternative approach, comparative quantification of label-free LC-MS^n proteomics has emerged. Quantification of protein expression using a label-free method can be achieved by two methods: (1) spectral counting where the frequency of peptide identification of a particular protein is used as a measure of relative abundance and (2) ion intensity, which uses the mass spectrometric chromatographic signal intensity of peptide peaks belonging to a particular protein. Label-free approaches are applicable to all types of biological samples and are not limited by the source of the sample, the number of samples, or the number of time points to be compared. Spectral counting is based on the rationale that an increase in protein abundance will result in an increase in the number of product ion spectra (spectral count) collected for peptides from that protein (Kota and Goshe 2011). However, spectral counting performs poorly with low abundance proteins and does not take into account posttranslational modifications (PTMs) (Schulze and Usadel 2010). Such a label-free 'shotgun' proteomic approach identified approximately 3500 proteins in mature *Arabidopsis* pollen, and the integration of obtained proteomic data with published transcriptomics data sets resulted in more than 500 proteins that were not previously identified in mature pollen (Grobei et al. 2009). Additionally, this analysis revealed that proteins of the categories transcription, cell cycle and DNA processing are underrepresented in mature *Arabidopsis* pollen supporting the old idea that transcription is not essential for pollen tube growth (see Mascarenhas 1975). As a first comprehensive as well as detailed shotgun pollen proteome analysis in a non-model organism, namely, *Lilium longiflorum*, more than 270 membrane and membrane-associated proteins of different organelle membrane fractions were identified at five physiologically important time points for pollen germination and tube growth (Pertl et al. 2009). Identified proteins were mainly involved in cytoskeleton turnover, carbohydrate and energy metabolism, as well as ion transport, membrane/protein trafficking, signal transduction, stress response

and protein biosynthesis. In an iTRAQ-based comparative quantitative proteomic approach in lily (*Lilium davidii*) focussing on plasma membranes of pollen grains and pollen tubes, more than 220 integral and membrane-associated proteins were identified (Han et al. 2010). Only 14 proteins showed a differential expression pattern in the plasma membrane during the transition from pollen grains to pollen tubes, but these proteins are mainly involved in signalling, transport and membrane trafficking.

In addition to internal compartments, the apoplast of plant tissue, which includes the cell wall matrix and the intercellular space, has some distinct functions such as growth regulation, sustaining skeleton, homeostasis of the internal environment and transportation route (Sakurai 1998). However, detailed information about the functions of apoplastic proteins in pollen germination and during pollen tube growth is scarce. Most knowledge comes from screening of mutants, in which identified proteins were mainly involved in adhesion, hydration and maintenance of pollen tube growth (Cheung and Wu 2008; Mayfield and Preuss 2000; Jiang et al. 2005). To gain a deeper insight into the function of apoplastic proteins, Ge and co-workers (Ge et al. 2011) analysed the apoplastic proteome of mature and germinated *Arabidopsis* pollen grains by 2D DIGE and LC-MS/MS. 103 spots were significantly differentially expressed after pollen germination, and 98 spots (representing 71 proteins) were identified. Of these, 50 proteins were found to be apoplastic and mainly involved in cell wall modification and remodelling, protein metabolism and signal transduction. As the apoplast is the first subcellular component of the pollen to be exposed to signals from the surrounding stigma or pistil tissues, it is hypothesized to have important roles in signal transduction. During pollen tube growth, new cell wall has to be synthesized quickly, and, on the other side, the cell wall of the stigma and the transmitting tract then have to be loosened. Additionally, the rapid growth requires a fast turnover of proteins. Taken together, these results from proteome studies highlight the importance of signal transduction, energy metabolism, transport of ions and nutrients, protein biosynthesis as well as cell wall biosynthesis and endocytosis in maintenance of polarized tip growth.

11.2.2 Pollen Allergomics

Since pollen allergens are mainly proteins of the pollen apoplast and pollen coat or associated with the pollen cell wall, much information on pollen proteins and pollen cell wall proteomes can be found in studies of pollen allergens. However, these data were collected in respect to allergic reactions in humans and are, therefore, only of limited use for plant physiologists but still provide a convenient source of sequence information, especially for non-sequenced pollen species. Because pollen allergy affects about 25% of the population in industrialized countries, and numbers are still increasing, the determination of the allergenic potential of several pollen proteins is of public interest. Allergies are immunological disorders, characterized

by immune responses directed against normally harmless environmental substances, such as airborne grass or tree pollen, as well as weed pollen species. Sensitive people then produce IgE antibodies that lead to allergic symptoms, ranging from rhinitis to bronchial asthma (Petersen et al. 2006).

Plant allergens have various roles: some serve as storage proteins, some are involved in plant defence and lipid transport, and others act as protease inhibitors, actin-binding proteins or structural proteins. Pollen allergens are classified according to their biochemical structure and immunological reactivity. For detection of allergens in plants, proteomic techniques that target allergens, i.e. allergomics, have become powerful tools for comprehensive allergen analysis (Nakamura and Teshima 2013, see also Chap. 10). Compared to conventional methods, e.g. classical isolation methods of single proteins, proteomics has clearly accelerated identification of multiple allergens in pollen. Important issues regarding allergen identification and quantification are the sensitivity and the specificity of the applied method. Mass spectrometry-based approaches are useful to investigate the various biological questions related to pollen allergy and have some advantages over routinely used methods to detect and quantify allergens. For example, ELISAs, where IgG antibodies from immunized animals are used for allergen detection, are only as good as the specificity of the used antibodies. On the one hand, an IgG antibody may not recognize all isoforms of a particular allergen or, on the other hand, may also recognize isoforms of the allergen that do not trigger an immune response (Schenk et al. 2010). Mass spectrometry methods have the advantage that they detect the allergens directly and independently of antibody specificity, namely, unbiased. One limitation of allergomics is that it is impossible to know the allergenic potential of the novel, identified IgE-binding proteins, because not all IgE-binding proteins cause allergic responses (Nakamura and Teshima 2013).

To analyse putative allergenic pollen proteins by mass spectrometry, special care has to be taken about the source of pollen material and the way how the material is prepared. Allergen expression levels have been shown to vary depending on genetic backgrounds and environmental factors during pollen development. For identification of allergenic pollen proteins, pollen grains are often incubated in solutions to release the allergenic proteins. These fractions have to be denoted as ‘pollen exudates’ or ‘pollen diffusates’ to distinguish them from pollen extracts which are prepared by damaging the pollen grains, and therefore, the high amounts of intracellular proteins would cover the less abundant allergens (Hoidn et al. 2005; Pertl-Obermeyer and Obermeyer 2013). Allergens are often members of multigene families, whose genes encode for similar isoforms that differ only by a few amino acids (Schenk et al. 2010). Different isoforms of pollen allergens often vary in their immune reactivity as shown for the major birch allergen Bet v 1 (Ferreira et al. 1996) with hypoallergenic isoforms that can be used as putative valuable tools for immunotherapy. Their allergenic potential is not determined by the total allergen content but by the quantity of the hypoallergenic isoform or the quantity of their specific peptides. Mass spectrometry methods can determine their quantities by the use of stable isotope-labelled internal standards or by label-free approaches, i.e. comparison of signal intensity or spectral counting. Such a label-free MS procedure

was used to screen pollen of different birch species and varieties for identification of hypoallergenic birches (Silva et al. 2005). Nevertheless, the main drawbacks for identification of allergens by mass spectrometry are the still incomplete databases used for peptide search.

11.3 Pollen Membrane Proteome

The ultimate challenge of membrane proteomics is to develop a method that allows extracting, separating and identifying the often low abundant peripheral and integral membrane proteins out of the highly heterogeneous mixture of proteins within a cell. Membrane proteins vary in abundance and show extremely different physicochemical properties, e.g. different hydrophobicity or extreme isoelectric points (p*I*), which makes it difficult to separate them by chromatography techniques. Therefore, an analysis of membrane proteins very often requires the combination of various extraction and fractionation strategies.

In pollen, the tip-focused growth is regulated by a network of cellular processes, including a highly active and dynamic membrane/protein trafficking system including endo- and exocytosis. For example, plasma membrane (PM) proteins move through the endoplasmic reticulum (ER) and the Golgi apparatus before they reach their sites of action and are recycled via endocytosis. Therefore, only a comprehensive view of many membrane compartments reflects the dynamic subcellular distribution of membrane proteins. Although pure membrane fractions can be achieved by aqueous two-phase partitioning systems, it gives only limited insights because only a single membrane compartment can be isolated during one preparation and some subcellular organelle membranes are still difficult to purify (Larson 1983). To overcome these limitations, separation of endomembrane and plasma membrane vesicles by (dis)continuous density centrifugation (Fig. 11.1) allows analysing the dynamics of protein abundance in different cellular compartments (Obermeyer et al. 1996; Pertl et al. 2005, 2009). One of the major advantages in using a (discontinuous) sucrose step gradient is that all organelle fractions from different physiological states during pollen grain germination and tube growth can be separated and enriched in a single preparative step for subsequent mass spectrometry analysis.

To assign the interphases of the step gradient to organelle membrane-enriched fractions, the distribution of typical organelle marker proteins across the gradient has to be analysed (Fig. 11.2). Therefore, well-characterized membrane proteins with known location to different subcellular compartments, such as mitochondria, endoplasmic reticulum, Golgi apparatus, plasma membrane and vacuole, have to be chosen. For example, known residents of the plasma membrane are members of the PM H^+ ATPase family or plasma membrane-localized aquaporins (PIPs). Markers for the endoplasmic reticulum are members of ER-localized Ca^{2+}-ATPases (ECAs) and ER-localized chaperons such as calreticulins (CRTs) and members of the HSP70 family (BIPs, Dunkley et al. 2006; Nikolovski et al. 2012). F-type ATPases

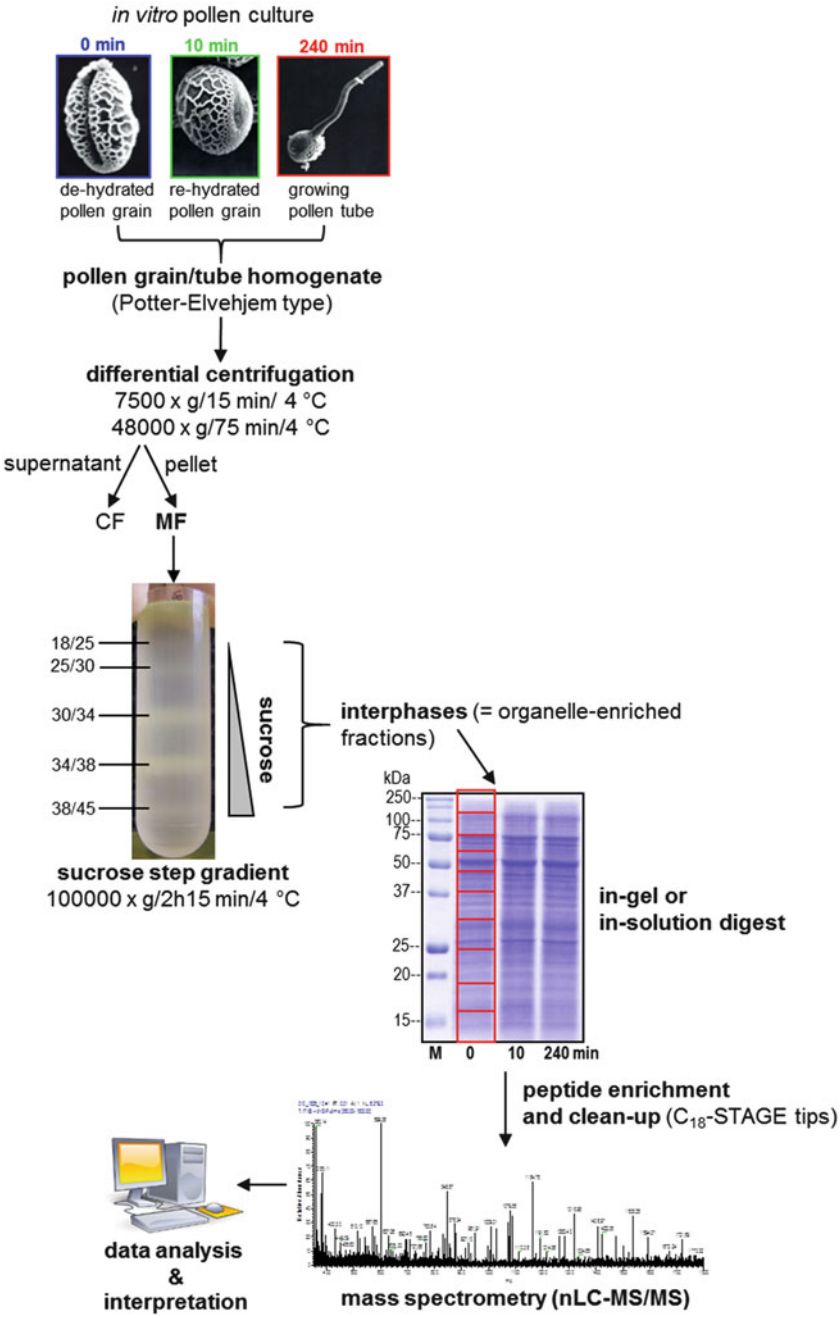

Fig. 11.1 Experimental design of a label-free shotgun proteomic workflow. A microsomal fraction (*MF*) was prepared from lily (*Lilium longiflorum*) pollen grains and tubes incubated for various times in germination medium representing specific states for lily pollen. The MFs obtained from the different time points were further separated by discontinuous sucrose density centrifugation resulting in interphases 18/25%, 25/30%, 30/34%, 34/38% and 38/45%. Proteins of each interphase and each time point were separated by SDS-PAGE or used directly (in-solution digest), and trypsin-digested proteins were analysed by mass spectrometry (nLC-MS/MS)

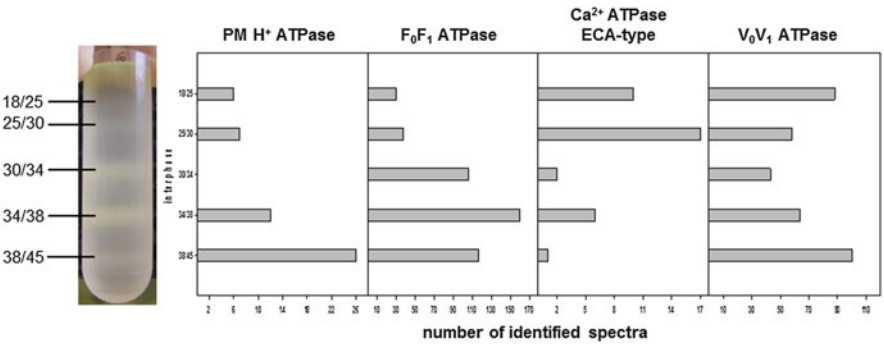

Fig. 11.2 Distribution of typical organelle marker proteins in the discontinuous sucrose gradient. Most spectra identifying the PM H^+ ATPase were found in interphase 38/45; for the F_0F_1 ATP synthase, most spectra were identified in interphase 34/38. For the ER-localized Ca^{2+}-ATPase, highest number of spectra were found in interphase 25/30, and in interphases 18/25 and 38/45, most spectra for the V_0V_1 ATPase were identified (Pertl et al. 2009)

and cytochrome c oxidase II, and ADP-ribosylation factors (ARFs) as well as glycosyl transferases are markers for mitochondria and Golgi-derived membranes, respectively. To assign proteins to vacuole-enriched fractions V-ATPases, the H^+-PPase or tonoplast-localized aquaporins (TIPs) are used as marker proteins (Pertl et al. 2009; Gattolin et al. 2009). A discontinuous sucrose gradient combined with marker analysis was used in a 'shotgun' proteomic approach to identify membrane and membrane-associated proteins from lily pollen grains and pollen tubes (Pertl et al. 2009). Thereby, an increase in abundance of proteins involved in cytoskeleton, carbohydrate and energy metabolism as well as ion transport before pollen grain germination was observed (10–30 min), whereas proteins involved in membrane/protein trafficking, signal transduction, stress response and protein biosynthesis decreased during early stages of pollen grain germination/tube growth (<30 min) followed by an increase just after tube germination (60 min, Fig. 11.3). Time-dependent changes in protein abundance may depict the up- and downregulation of a biological process during characteristic phases of pollen action. The changes in peptide/protein abundance between 10 and 30 min after starting the pollen culture emphasize the importance of this time period for initiating pollen germination. Although no visible signs of germination are observable during the first 30 min, a number of processes were postulated to take place inside the pollen grain: adjustment of ionic concentrations and pH, building up of a membrane potential and osmotic gradient, reorganization of the organelle membranes and the cytoskeleton, respiration and energy supply. These processes are well mirrored by the dynamic changes in protein abundances at this time, for example, the fast increase in the number of identified peptides involved in cytoskeleton organization and turnover or in energy metabolism and in ion transport. Proteins involved in some other biological processes, such as protein biosynthesis and membrane/protein trafficking, became more abundant after 30 min when the pollen grain starts to germinate,

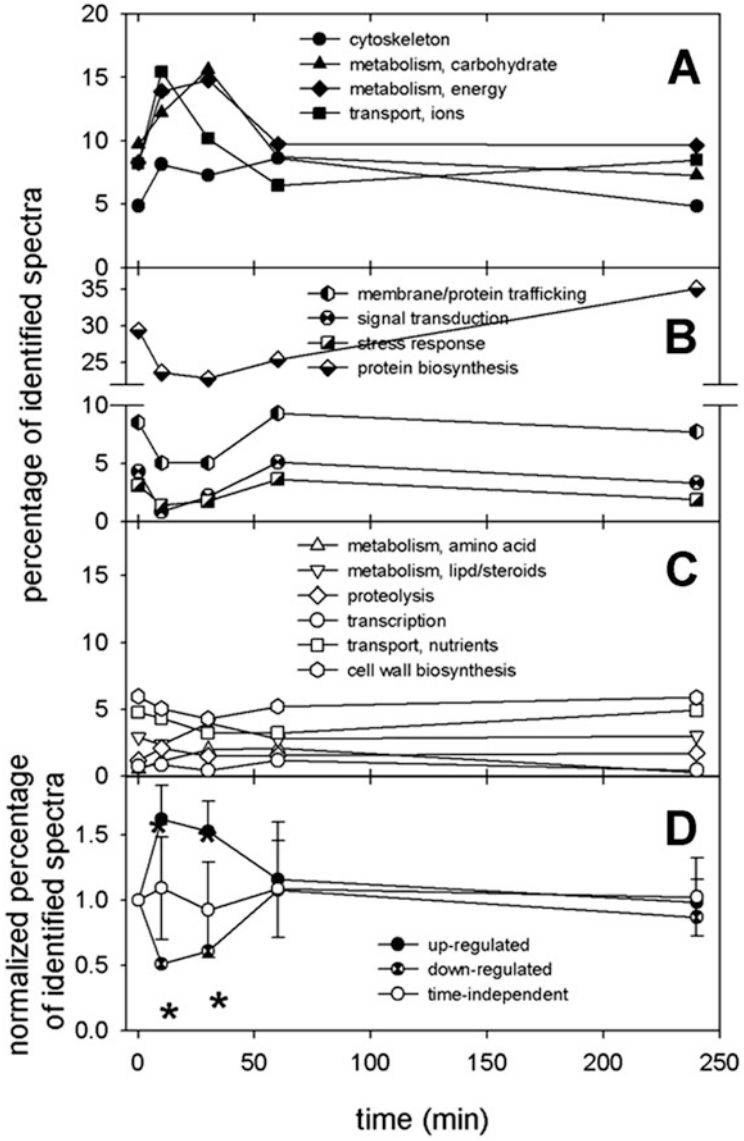

Fig. 11.3 Time dependence of functional classes during pollen grain germination and tube growth. Temporal changes in protein abundance could be observed for some biological processes during pollen grain germination and tube growth. The relative numbers of identified spectra were plotted against time. (**a**) Biological processes that show a higher abundance of peptides (spectra) in the first 30 min are 'cytoskeleton', 'carbohydrate metabolism', 'energy metabolism' and 'ion transport'. (**b**) The amount of peptides/spectra of the functional categories 'membrane/protein trafficking', 'signal transduction', 'stress response' and 'protein biosynthesis, folding and modification' was lower during the first 30 min and increased after rehydration and germination of the pollen grains. (**c**) All other biological relevant processes did not show significant changes during the 240 min of pollen grain in vitro culture. (**d**) The up- and the downregulated processes at time points 10 and 30 min were significantly different from their time point 0 min (Pertl et al. 2009)

thus reflecting the need of a constant supply of new proteins and the synthesis and delivery of membranes to the germination site/growing tube tip.

11.3.1 Membrane Proteome in Signalling and Stress Response

During their development or during the progamic phase, pollen grains can be exposed to stress conditions, e.g. like changes in metabolic activity or in response to desiccation. The pollen response to these stresses is accompanied by the activation of many genes and protein expression involved in stress perception and in transduction of stress signals, which results in further modulation of gene activity or protein expression. In tomato pollen, a member of aldehyde dehydrogenase (ALDH) gene family localized to plastids was identified. ALDHs are expressed during oxidative stress and are known to be critical in detoxification of aldehydes generated as a product of different cellular processes (Paul et al. 2016). In this study, also two plasma membrane-localized glycerophosphodiester phosphodiesterase-like family of proteins (GDPL) were identified, which play pivotal roles in conditions of inorganic phosphate (P_i) starvation. In tomato as well as in lily pollen, members of the dehydration-responsive protein-like family (DRP) localized to different cellular sub-compartments have been identified (Paul et al. 2016; Pertl et al. 2009). LEA (late embryogenesis abundant) proteins provide protection from dehydration and play important roles in pollen function at maturity and during subsequent germination and tube growth. Many LEA proteins are induced by cold or osmotic stress or by exogenous ABA and were found in *Arabidopsis* pollen (Sheoran et al. 2006; Grobei et al. 2009) and in lily pollen (Pertl et al. 2009). As stated, the external stress signals have to be sensed and transduced to the cell interior. In this context, PM-/ER-localized phosphoinositide phosphatases (PIPase) have been found. These proteins modulate the phosphoinositide (PI) level within cell membranes, thereby regulating various signal transduction processes. Moreover, membrane-associated proteins such as 14-3-3 proteins, phospholipase D, calmodulin and heterotrimeric G-protein complexes, all of them well known to play important roles in stress signal transduction, were identified (Dai et al. 2006; Pertl et al. 2009). 14-3-3s are major regulators in plant development and stress physiology, typically via a phosphorylation-dependent interaction with target proteins (Pertl et al. 2011; van Kleeff et al. 2014). Phospholipase D (PLD) hydrolyzes membrane phospholipids for the production of phosphatidic acid (PA) which play important roles in signal transduction via a proposed activation of the PM H^+ ATPase activity (Shen et al. 2011; Potocký et al. 2014). Calmodulin is a multifunctional receptor protein for intracellular Ca^{2+}, and heterotrimeric G-proteins are conserved signal-transducing proteins in the plasma membrane involved in tip growth (Ma et al. 1999). Recently, it was shown that G-protein subunit α participates in pollen germination through modulation of the hyperpolarization-activated Ca^{2+} channel in the plasma membrane of *Arabidopsis* pollen (Wu et al. 2007).

The accumulation of intracellular signalling molecules causes the modulation of enzyme activities or gene/protein expression and in growing pollen tubes in determination of the growth direction. As the growing pollen tube has to be guided to the ovule for fertilization, a meticulous communication between male and female organs and tissues is essential (see also Chap. 8). In tomato pollen (*Solanum lycopersicum*), two plasma membrane-localized LRR-RLKs (leucine-rich repeat receptor-like kinases), *Le*PRK1 and *Le*PRK2, were identified which are involved in the regulation of pollen germination and pollen tube growth (Löcke et al. 2010). They form heterodimers and are components of a multimeric protein complex in which the *Le*PRKs presumably have a key role in transducing exogenous signals through the plasma membrane of the growing pollen tube. Receptor-like kinases have been identified in various plant species and in *Arabidopsis*, the RLK family includes >600 members, with the LRR-RLKs constituting the largest group (Osakabe et al. 2013). Receptor-like kinases (RLKs) and LRR-RLKs have been identified in lily pollen (Pertl-Obermeyer et al. 2014) and RLKs in canola pollen (Sheoran et al. 2009) and in *Arabidopsis* pollen (Holmes-Davies et al. 2005). Again, proteome analysis of the entire membrane compartment or a particular signal transduction pathway enables the researcher to investigate the dynamics of all components of the pathway in a single experiment instead of single components in many experiments.

11.3.2 Membrane Proteome in Cell Wall Biosynthesis

The complex plant cell wall structure is built and maintained by diverse proteins involved in cell wall synthesis, modification and secretion. The major structural and functional constituents of the walls are hemicelluloses, cellulose, pectin and lignin, whose relative content varies depending on the species, tissue and cell development and growth stages (Kim and Brandizzi 2014). The Golgi and plasma membranes are the two main sites where non-lignin cell wall constituents are synthesized.

Upon contact with the stigma, the pollen grain has to transmit the pistil tissue and grow to the ovary to deliver the sperm cells for fertilization (Heslop-Harrison 1987). For all of these functions, the pollen tube cell wall plays an important regulatory and structural role (see also Chaps. 3 and 8). The secondary cell wall layer of pollen tubes consists of two layers, the outer formed by pectin and the inner formed by callose. Depending on the species, cellulose microfibrils have been found to be associated either with the outer pectic or with the inner callosic layer (Chebli et al. 2012). The pollen cell wall provides structural support during development, and its functional integrity has to be maintained during pollen tube growth. Several enzymes involved in cell wall synthesis like subunits of the PM-localized large multimeric cellulose synthase complexes (CESA), the callose synthase (CALS) and members of the pectin (methyl)esterase family have been identified in tomato, lily and *Arabidopsis* pollen (Paul et al. 2016; Pertl et al. 2009; Holmes-Davies et al. 2005; Dai et al. 2006). Additionally, cell wall component modifying proteins, such

as UDP-glucose-, UDP-galactose-, UDP-glucuronate- and UDP-xylose-epimerase, are also involved in cell wall synthesis and were identified in these pollen proteome studies.

11.3.3 Membrane Proteome in Protein Trafficking and Protein Biosynthesis, Folding and Modification

The secretory pathway consists of numerous functionally interlinked organelles. The first organelle of the secretory pathway is the endoplasmic reticulum (ER) in which proteins are synthesized and assembled for export to the Golgi apparatus. The Golgi apparatus then collects membranes and luminal cargo from the ER for further processing and sorting to distal compartments which include the trans-Golgi network (TGN), vacuoles and the plasma membrane (Kim and Brandizzi 2014). The secretory pathway is of importance to pollen especially for pollen tube elongation. Tip-directed trafficking of membrane vesicles and proteins involves pairing of SNAREs (Soluble N-ethylmaleimide-sensitive factor attachment protein receptors) to facilitate the membrane fusion. Functional classification divides SNAREs into vesicle-associated and target membrane-associated SNAREs (v- and t-SNAREs). Alternatively, the structural classification groups SNAREs as Q- and R-SNAREs owing to the occurrence of either a conserved glutamine (Q) or arginine (R) residue in the centre of the SNARE domain (Tyrrell et al. 2007). Generally, t-SNAREs correspond to Q-SNAREs, and v-SNAREs correspond to R-SNAREs. In that context, SNAREs and SNARE-associated proteins were identified in lily, tomato and *Brassica napus* pollen (Pertl et al. 2009; Paul et al. 2016; Sheoran et al. 2009). Additionally, ADP-ribosylation factor (Arf) and secretion-associated and Ras-related protein (Sar) are major regulators of vesicle biogenesis in intracellular trafficking controlling the assembly of coat protein to facilitate budding of COPI and COPII vesicles, respectively (Memon 2004), and were identified in tomato, *Arabidopsis* and lily pollen (Paul et al. 2016; Holmes-Davies et al. 2005; Pertl et al. 2009). Rab GTPases are known to associate with various organelle membranes and assist vesicle trafficking between ER and Golgi, trafficking of secretory vesicles and exocyst formation (Cheung et al. 2002; de Graaf et al. 2005; Hala et al. 2008), and in lily pollen, Rab-type small GTPases could be detected in pollen grains and growing pollen tubes (Pertl et al. 2009), as well as in tomato pollen (Paul et al. 2016) and in *Arabidopsis* pollen (Holmes-Davies et al. 2005). As expected GDP dissociation inhibitor proteins, GTP-binding proteins, clathrin, clathrin adaptor proteins, COP family proteins and dynamins, all involved in protein and membrane trafficking, were identified in pollen.

ER-localized members of the calreticulin family, which act as calcium-binding chaperones promoting protein folding, oligomeric assembly and quality control in the ER, were identified in several pollen proteome studies in *Arabidopsis* (Holmes-Davies et al. 2005; Noir et al. 2005; Sheoran et al. 2006; Zou et al. 2009), lily (Pertl

et al. 2009), tomato (Sheoran et al. 2007; Paul et al. 2016), canola (Sheoran et al. 2009) and rice (Dai et al. 2006, 2007a). Proteins involved in protein biosynthesis, such as ER-associated ribosomal proteins, elongation factors and prohibitins, were also found. Prohibitins play a crucial role in mitochondrial biogenesis, protein processing and transcriptional control (Van Aken et al. 2010). BiPs (luminal binding proteins) are ER-localized members of the HSP70 family and are known to bind to misfolded, underglycosylated or unassembled proteins whose transport from the ER is blocked. Oligosaccharyltransferase (OST) is a membrane protein complex in the ER that transfers a sugar oligosaccharide from dolichol to nascent proteins. All these proteins, including some other sugar transferases or disulphide isomerases as well as different heat shock protein family members and chaperones, which are involved protein folding and modification, were identified in these pollen proteome studies.

11.3.4 Membrane Proteome in Ion and Nutrient Transport

The plant plasma membrane (PM) is the outermost membrane of the cell that functions as interface with the extracellular environment for exchange of information and substances. The PM is a dynamic structure. A highly active vesicle transport to and from cytoplasmic organelles allows a rapid modification of the plasma membrane composition in response to stimuli by triggering downstream signalling events. The composition of the plasma membrane very often varies with the cell type, developmental state and environment, resulting in several combinations of different protein classes such as transporters, channels, receptors and signalling components.

In general, the PM H^+ ATPase is an important housekeeping enzyme generating an electrochemical H^+ gradient across the plasma membrane for the transport of nutrients and ions into the cell through various channels or carrier proteins (Palmgren 2001). Pollen grains of many species can be easily cultured in diverse synthetic culture media, and during in vitro cultivation, an acidification of the germination medium could be observed due to H^+ extrusion from the pollen grains (Lang et al. 2014 and references therein). It was demonstrated that the PM H^+ ATPase is responsible for the medium acidification (Pertl et al. 2010). In accordance to physiological studies showing an effect of the fusicoccin-activated or the vanadate-inhibited PM H^+ ATPase on pollen grain germination frequencies (Rodriguez-Rosales et al. 1989; Fricker et al. 1997; Pertl et al. 2001; Sun et al. 2009), the importance of the PM H^+ ATPase during the early state of germination, e.g. transition from the 'quiescent' to the hydrated state of pollen grains, is supported by the increase in PM H^+ ATPase proteins within the first 10 min of pollen grain culture (Pertl et al. 2009). Additionally, it has been demonstrated that in pollen grains of *Lilium longiflorum*, osmoregulation occurs via modulation of the PM H^+ ATPase activity (Pertl et al. 2010). An increase in PM H^+ ATPase activity and an increase in membrane-associated 14-3-3 proteins were detected upon hyper-osmolar treatment of pollen grains suggesting a modulation of its activity by phosphorylation and subsequent binding of 14-3-3 proteins. 14-3-3 proteins are well-characterized

modulators of the PM H^+ ATPase (Svennelid et al. 1999; Fuglsang et al. 1999; Maudoux et al. 2000). All studies showed a direct correlation between the PM H^+ ATPase activity and the pollen germination as well as tube growth and thus demonstrate an active PM H^+ ATPase as a prerequisite for successful pollen germination and tube growth. Surprisingly, besides lily pollen, proteins encoding a PM H^+ ATPase were only identified in proteome studies of tomato pollen (Paul et al. 2016) and of rice pollen (Dai et al. 2006). Compared to other organelle membranes, PM is less abundant, and therefore, the PM H^+ ATPase is easily missed in proteome analyses not using membrane-enriched fractions or 2D DIGE for protein fractionation.

Two other H^+-translocating proteins, the V-type ATPase and the H^+ pyrophosphatase (H^+ PPase), are more abundant and could be identified in several pollen species. Both proteins are known to reside in the tonoplast and endomembrane compartments of pollen (Mitsuda et al. 2001; Padmanaban et al. 2004; Dettmer et al. 2005). V-ATPase subunits were found in *Arabidopsis* pollen (Noir et al. 2005; Holmes-Davies et al. 2005; Sheoran et al. 2006; Zou et al. 2009), in canola pollen (Sheoran et al. 2009), in rice pollen (Dai et al. 2006, 2007a) as well as in tomato and lily pollen (Paul et al. 2016; Pertl et al. 2009). The overall high abundance of these subunits reflects the functional relevance of the formation of vacuoles during pollen germination and their role in acidifying the vacuole lumen. H^+ pyrophosphatases were identified in pollen of lily, tomato and *Arabidopsis* (Pertl et al. 2009; Paul et al. 2016; Noir et al. 2005; Sheoran et al. 2006), but not in rice pollen. These tonoplast-localized proton pumps play a central role in maintaining proton homeostasis.

Another group of proteins known to be essential for pollen development are Ca^{2+}-ATPases. They have a high affinity for Ca^{2+} (0.1–2 μM) (Sze et al. 2000) and are members of the P-type ATPase superfamily (Axelsen and Palmgren 1998). In plants, Ca^{2+} -ATPases can be subdivided into two phylogenetic groups, the autoinhibited-type Ca^{2+}-ATPases (ACA) and the endoplasmic reticulum-type Ca^{2+}-ATPases (ECA), which both are regulated by calmodulin (CaM). Both ECAs and ACAs have been found at the PM, tonoplast and endoplasmic reticulum (Sze et al. 2000). It has been shown that *A. thaliana* ACA7 is a plasma membrane protein that has an important role during pollen development, possibly through regulation of Ca^{2+} homeostasis (Lucca and León 2012). In *Arabidopsis* ACA9 is expressed almost exclusively in pollen, and knockout of ACA9 leads to significant impairment of pollen tube growth (Schiott et al. 2004). Surprisingly, ACAs and ECAs were only detected in proteome studies of tomato pollen (Paul et al. 2016) and lily pollen (Pertl et al. 2009).

Voltage-dependent anion channel (VDAC) is the most abundant protein in the mitochondrial outer membrane which mediates the transport of metabolites (succinate, citrate, malate, ATP and ADP) between mitochondria and the cytoplasm. VDAC proteins belong to a small multigene family: the *Arabidopsis* genome contains five putative VDAC genes, and in poplar (*Populus trichocarpa*) ten genes are found. Microarray assays showed that AtVDAC2, AtVDAC3 and AtVDAC4

are expressed in pollen (Homblé et al. 2012), and recently, it has been shown that VDAC members are also important for vegetative and reproductive growth, while T-DNA knockout mutants of AtVDAC2 showed retarded growth and abnormal pollen development (Tateda et al. 2012). The varying number of genes may indicate a diversity of VDAC functions in plants. Several studies demonstrated that VDAC can also be found in non-mitochondrial cell membranes, such as in the membrane of peroxisomes, glyoxysomes and chloroplasts as well as in the plasma membrane (Homblé et al. 2012 and references therein). VDAC family members were identified in proteome analyses of tomato pollen (Paul et al. 2016), rice pollen (Dai et al. 2006, 2007b), mature *Arabidopsis* pollen (Holmes-Davies et al. 2005) and lily pollen (Pertl et al. 2009).

The uptake of K^+ is important for tube growth by probably balancing the osmotic potential of the cytosol and the turgor pressure during rapid tube elongation (Benkert et al. 1997; Winship et al. 2010). Generally, K^+ influx is caused by voltage-gated and acidic pH-sensitive inward rectifying K^+ channels (Griessner and Obermeyer 2003). So far, SPIK (=AKT6, AT2G25600) from *A. thaliana* (Mouline et al. 2002) and an AKT1-like K^+ channel (LilKT1) from *L. longiflorum* are the only inward rectifying K^+ channels that have been identified in pollen (Safiarian et al. 2015).

Compared to cytosolic or soluble proteins, the listed examples of identified membrane proteins and transporter are less abundant in the pollen, and their peptides are often missed during mass spectrometry analysis. This supports the view that appropriate membrane preparation and enrichment methods are necessary to investigate membrane proteins (Pertl-Obermeyer and Obermeyer 2013). More often, opposing techniques might have to be applied to identify all, even the lowest abundant membrane proteins by proteomics, to obtain a comprehensive image on the dynamics of membrane proteins in an organism.

11.3.5 Membrane Protein Complexes in Pollen

In addition to identification of single proteins, the knowledge about protein complexes and protein interaction partners becomes more and more important, especially for membrane proteins. Many examples showed that transmembrane signalling across the plasma membrane directly affects membrane proteins that are bundled in functional complexes. For instance, the interaction between LRR-receptor-like kinase BRI1 (brassinosteroid-insensitive 1) and BAK1(Serk3) (BRI1-associated kinase1 also known as somatic embryogenesis receptor-like kinase) is necessary to perceive brassinosteroid signals and transmit the signal to cytosolic components, and interaction between BAK1 and PM H^+ ATPases AHA1 and 2 together with cyclic nucleotide-gated channel CNGC17 is involved in transduction of phytosulfokine signals in *Arabidopsis* plants (Bücherl et al. 2013; Ladwig et al. 2015). Apart from molecular biology techniques to investigate protein-protein interactions, e.g. mbSUS, BiFC, etc., cross-linked and immunoprecipitated membrane protein complexes can be analysed by proteomic methods (Liu et al.

2015). So far, membrane protein complexes had been investigated in lily pollen only (Pertl-Obermeyer et al. 2014). Membrane proteins were rapidly cross-linked by glutaraldehyde, and complexes containing the PM H^+ ATPase were purified using immunoprecipitation with specific antibodies against the pollen proton pump. Interestingly, a large number of receptor kinases and calcium signalling proteins as well as 14-3-3 proteins are closely associated in the plasma membrane with the H^+ ATPase, thus forming a functional complex that is able to react as fast as possible to external signals. This rapid cross-linking strategy is quite new for plant research but has now also been used to identify functional PIP (aquaporin) complexes in *Arabidopsis* roots (Bellati et al. 2016). This rapid cross-linking techniques allows to 'freeze' the proteins/complexes in a specific state after an external signal and is method with a high future potential to investigate dynamics of membrane protein complexes in a system showing fast responses to external signals and a dynamic growth behaviour: the pollen tube.

11.4 Conclusion and Perspective

So far, in most of the pollen proteome studies, single proteins and protein families have been listed or identified, and the pollen proteome has been compared to the proteomes of other tissues to reveal pollen-specific proteins or protein isoforms. In some studies, the differences in the proteome between two or three states, e.g. pollen grains and tubes, were investigated. However, the germination and growth of pollen tubes is a highly dynamic process. Pollen grains have to transit from a quiescent to an active phase after landing on the stigma surface and the tubes change growth speed or growth direction from 1 min to the other. To understand these dynamic processes, a much higher time resolution for pollen proteome studies is necessary to catch the proteins in the act. In the moment, we are just at the beginning of using the power of proteomic techniques to understand the dynamics of pollen tube growth. Furthermore, biological processes are carried out by interactions between several biomolecules, and especially membrane proteins may form functional units which allow a fast propagation of signals from one molecule to the other. Therefore, not only the identification of proteins involved in tip growth is important but also to know their interacting partners at specific moments. It might turn out that the proteins in the plasma membrane may have different interaction partners or are involved in different functional protein complexes, just depending on the physiological process to be performed, e.g. during straight growth, the PM H^+ ATPase may interact with other proteins than during cutting a corner. Proteome studies with a high temporal and spatial resolution in combination with interactome studies are needed in the future to reveal the role of functional membrane protein complexes in tip growth.

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