Isobaric Tags for Relative and Absolute Quantification-based Comparative Proteomics Reveals the Features of Plasma Membrane-Associated Proteomes of Pollen Grains and Pollen Tubes from *Lilium davidii*

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Abstract

Mature pollen grains (PGs) from most plant species are metabolically quiescent. However, once pollinated onto stigma, they quickly hydrate and germinate. A PG can give rise to a vegetative cell-derived polarized pollen tube (PT), which represents a specialized polar cell. The polarized PT grows by the tip and requires interaction of different signaling molecules localized in the apical plasma membrane and active membrane trafficking. The mechanisms underlying the interaction and membrane trafficking are not well understood. In this work, we purified PG and PT plasma-membrane vesicles from *Lilium davidii* Duch. using the aqueous two-phase partition technique, then enriched plasma membrane proteins by using Brij58 and KCl to remove loosely bound contaminants. We identified 223 integral and membrane-associated proteins in the plasma membrane of PGs and PTs by using isobaric tags for relative and absolute quantification (iTRAQ) and 2-D high-performance liquid chromatography-tandem mass spectrometry. More than 68% of the proteins have putative transmembrane domains and/or lipid-modified motifs. Proteins involved in signal transduction, membrane trafficking and transport are predominant in the plasma-membrane proteome. We revealed most components of the clathrin-dependent endocytosis pathway. Statistical analysis revealed 14 proteins differentially expressed in the two development stages: in PTs, six upregulated and eight downregulated are mainly involved in signaling, transport and membrane trafficking. These results provide novel insights into polarized PT growth.

Introduction

The pollen (male gametophyte) of angiosperms consists of haploid vegetative cell and sperm cells enclosed in the vege-

tative cell, and is a key regulator in sexual reproduction and contributes to the genetic diversity of a population. Mature pollen grains (PGs) from most plant species are metabolically quiescent. However, once deposited onto stigma, they quickly

hydrate, germinate and give rise to vegetative cell-derived polarized pollen tubes (PTs). The PTs communicate with the pistil and grow directionally to deliver sperm cells into the embryo sac to initiate double fertilization.

Polarity is essential for generating diverse cell functions throughout development. The PT represents a specialized polarized cell, and it grows at a fast rate by the tip. For example, the lily PTs grow at an average of 200–300 nm/s *in vitro*, which exceeds that of neurite outgrowth in culture (Hepler et al. 2001). Compared with other plant cell systems commonly used, PT is a good model for the study of polar growth, because *in vitro*-cultured PTs maintain their natural polarity and grow synchronously and uniformly (Yang 2008). Therefore, the PT has been considered a unique model system for studying the establishment and maintenance of cell polarity, cell differentiation and cell-to-cell interaction (Hepler et al. 2001; Cheung and Wu 2008; Yang 2008).

Previous studies have revealed that Rho-related small guanosine-5'-triphosphatase (GTPase) from plants (ROP) plays a central role in polarized PT growth (Yang 2008). Apical plasma membrane (PM)-localized ROP regulates the tip-focused calcium gradient and apical F-actin (fragment actin filament) in the tip cytoplasm, which are required for targeting and fusing exocytosis-mediated vesicles to a defined tip PM domain established during germination (Krichevsky et al. 2007). Several other signal molecules, such as calcium sensors and receptor-like kinases, are implicated in the regulation of polarized PT growth, possibly by affecting ROP activity (Kaothien et al. 2005; Cole and Fowler 2006; Yoon et al. 2006; Zhang and McCormick 2007). Functions of these signal molecules depend on their localization in the tip PM. Loss of tip PM localization or lateral spread to a subapical region leads to severe depolarization (Yoon et al. 2006). The mechanisms regulating these signal molecules to a defined PM domain are largely unknown (Cole and Fowler 2006), although several studies have indicated that effectors of ROPs, such as RopGAP1 (Yang 2008) and RhoGDI (Klahre et al. 2006), are required for the apical PM localization of ROPs. Cytological studies suggested endocytosis as being crucial for PT growth by recycling membrane materials and taking in extracellular matrix components (Picton and Steer 1983; Cheung and Wu 2008). In animal cells, endocytosis plays a central role in signal transduction by regulating the polarized contribution of signal molecules in PM (Liberali et al. 2008). In PTs, the pathway seems to be involved in establishing and maintaining the asymmetric distribution of several proteins such as channel proteins and GTPases (Picton and Steer 1983; Cheung and Wu 2008). However, the precise roles of endocytosis in polarized PT growth are unknown, mainly because the identities of the pathways have yet to be defined.

The PM provides a selectively permeable barrier between the cell and its surroundings and has diversified biochemical and biological functions such as signal transduction, ion and metabolite transport, membrane trafficking, cell proliferation and death, and cell-to-cell interaction. Because the PM is a crucial site for polarized PT growth, proteomic analysis of the pollen PM can provide detailed information about its protein components and shed novel insights into the function of PM in PT growth. In recent years, proteomic analyses of pollen, some at the subcellular level (Honys et al. 2009; Pertl et al. 2009), have been carried out on the basis of 2-D gel electrophoresis and mass spectrometry (Kerim et al. 2003; Fernando 2005; Holmes-Davis et al. 2005; Noir et al. 2005; Dai et al. 2006, 2007;; Sheoran et al. 2007; Honys et al. 2009; Zou et al. 2009) or the "shotgun" system (Grobei et al. 2009; Pertl et al. 2009). However, to our knowledge, specific research on pollen PM has not been reported.

In the present study, we aimed to define the protein composition of the PM of PGs and PTs from *Lilium davidii* Duch., as well as the quantitative differences between the 2 PM proteomes to identify potential components in PM that are associated with polarized PT growth. We obtained highly enriched PM vesicles by aqueous polymer two-phase partitioning and used isobaric tags for relative and absolute quantitation (iTRAQ) tagging, as well as 2-D high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) to identify proteins in the lily PG and PT PM and determine the difference in protein expression between the PM proteomes.

Results

Isolation of PM from lily pollen

We isolated raw PM vesicles (RPMs) from *L. davidii* Duch. PGs and PTs from uniformly germinated PGs (**Figure 1**) and then treated RPMs with Brij 58 and KCl, which turns cytoplasmic-side-in vesicles into inside-out vesicles to remove proteins enclosed in the vesicles and/or unspecifically bound to the PM (Johansson et al. 1995; Alexandersson et al. 2004). Approximately 50% of proteins from RPMs were removed on comparing protein concentrations of RPMs and purified PM vesicles (PPMs). We examined the quality of the PPMs by western blot analysis with antibodies against marker proteins of different subcellular compartments, including P-type H^+-adenosine tri-phosphatase (ATPase) (VHA) in PM, OsRad21-3 in nuclei, COXII in mitochondria, V-ATPase in vacuoles, catalase in peroxisome, and cFBPase in cytoplasm (**Figure 2**). VHA was detected only in RPM and PPM fractions, with the PM marker protein greatly enriched in the PPM fraction. Antibodies against OsRad21-3, COXII, V-ATPase and catalase detected signals in the entire cell lysate and microsome fractions but not in RPM or PPM. The antibody against cFBPase detected a signal only in the entire cell lysate. Therefore, the PM isolation method we used can effectively enrich the PM proteins and remove contaminants.

Figure 1. Cytological characteristics of pollen grains and pollen tubes from *Lilium davidii* Duch.

(**A**) Mature pollen grains.

(**B**) Germinated pollen grains and pollen tubes.

(**C**) A representative pollen tube. Scale bar = 50 μm in A to C.
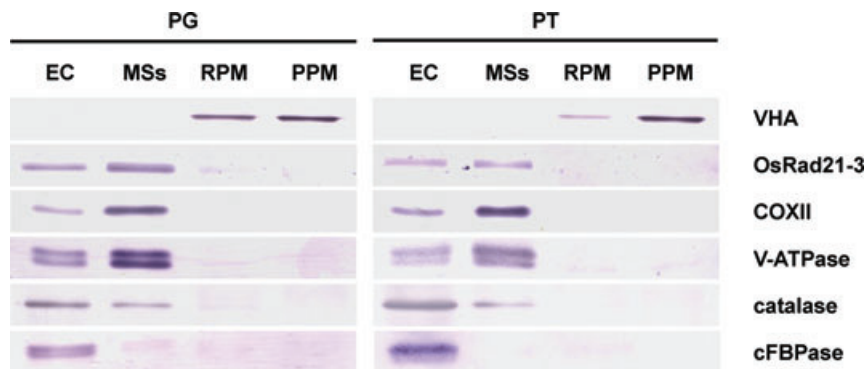

Figure 2. Immunoblot analysis of the purified plasma membrane.

Proteins from the entire cell lysate (EC), microsomes (MSs), raw plasma membrane vesicles (RPM), and purified plasma membrane vesicles (PPM) prepared from pollen grains (PGs) and pollen tubes (PTs) were separated on 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunodetected with rabbit polyclonal antibodies against P-type ATPase (VHA; PM marker), OsRad21-3 (nucleus marker), COXII (mitochondrial marker), V-ATPase subunit E (vacuole marker), catalase (peroxisome marker), and cFBPase (cytosolic marker). Five microgram proteins were loaded in each lane.

Protein identification

The PM proteome is relatively complex and dynamic; thus, direct application of LC-MS/MS for identification of PM proteins would lead to low efficiency. Prefractionated samples combined with subsequent LC-MS/MS can achieve higher efficiency in identifying PM proteins (Morel et al. 2006). Here, we fractionated the iTRAQ-labeled PM peptide mixtures by strong cation-exchange (SCX) chromatography into 12 fractions to reduce the sample complexity (Figure S1). Each fraction was then analyzed on nanoflow reverse-phase high-performance LC coupled with nanospray quadruple time-of-flight MS. Altogether, we identified 360 unique proteins in the PG and PT

PM samples (Table S1). For proteins with a single peptide (94 proteins), the MS/MS spectrum of the peptide was manually inspected and carefully identified (Figure S2). Searching against a concatenated database allowed for the calculation of false discovery rates of 4% at the protein level for these experiments.

Of the 360 identified proteins, 137 belonged to proteins involved in translational apparatus (91 ribosomal proteins and 17 components of translational initiation factor [eIF] and elongation factor [EF]) and DNA/RNA binding (29) (Table S2), some of these proteins were frequently identified in other PM proteomes (Table S2). These proteins were generally considered to associate with PM directly or via the cytosketelon (see Discussion section) and were excluded from further analysis. We analyzed

PM localization information for the remaining 223 identified proteins using online software tools and knowledge of known PM proteins. By a BLAST search of the *Arabidopsis* database (http://www.arabidopsis.org/index.jsp), which contains information on PM proteins identified from plant vegetative tissues and cultured cells (Alexandersson et al. 2004; Marmagne et al. 2004; Marmagne et al. 2007), we found 119 (53.4%) of the 223 proteins assigned to the PM and/or cell wall from previous proteomic and/or cytological studies (Table S2). The HMMTOP program, which is useful to predict proteins spanning the membrane in plants (Marmagne et al. 2007), predicted 116 (52.0%) of the 223 proteins to have at least one transmembrane (TM) domain (one TM domain for 51 proteins, and two or more for 65 proteins) (**Figure 3**, Table S2). The prediction was supported in part by several known proteins spanning the membrane, such as OsWAK receptor-like protein (OsWAK-RLP), inorganic pyrophosphatase and PM ATPase. Therefore, these proteins represent potential PM proteins.

Protein posttranslational modification by lipid moieties is important in regulating protein localization to the PM. The small GTPase is a well-known example. Almost all Rab proteins isolated to date have one or two isoprenylated cystine residues in their carboxyl terminus, which is essential for

membrane binding and interaction with regulatory molecules. For instance, Rab Ara6 is myristoylated and palmitoylated to be anchored to the membrane (Ueda and Nakano 2002). The glycosylphosphatidylinositol (GPI) anchor, myristoylation, prenylation and palmitoylation sites are indicative of protein PM localization. Our protein posttranslational modification analysis showed 15 proteins (6.7%) with myristoylation sites, four (1.8%) with prenylation sites, six (2.7%) with GPI anchor sites and 74 (33.2%) with palmitoylation sites (**Figure 3A**, Table S2). In total, 152 (68.2%) of the 223 proteins were predicted to have TM domains or one or more lipid-modified motifs, or both (**Figure 3B, C**, Table S2).

Of the 152 proteins with membrane-localizing features, 77 were known PM proteins from the known 119 PM proteins described above, which represented 64.7% (77/119) of the dataset (**Figure 3C**, Table S2). Thus, the two features we used represent good parameters for predicting the PM localization of proteins in lily. Altogether, our analysis revealed that 194 of the 223 proteins (87%) appeared to localize in or to associate with the PM (**Figure 3C**).

These data clearly indicate the high enrichment of PM proteins. The remaining 29 proteins do not have available information related to PM localization, but some are components of protein complexes, such as clathrin, adaptin, dynamin and SH3 domain proteins, and some contain domains involved in protein interaction, such as the WD40 domain (Table S2). So their PM association may be due to interaction with other proteins. However, as encountered in several PM proteomic studies, minor contamination from other organelles or the cytosol cannot be completely excluded (Alexandersson et al. 2004; Marmagne et al. 2004; Natera et al. 2008).
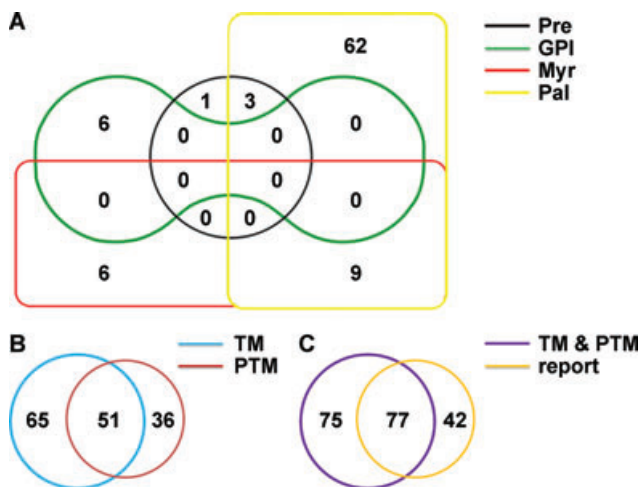

Figure 3. Edwards-Venn diagrams showing the distribution of proteins with different features.

(**A**) Overlapping of proteins with lipid-modified motifs: Myr (15), Pal (74), Pre (4) and GPI (6).
(**B**) Overlapping of proteins in A (posttranslational modification, PTM) with those with transmembrane domains (TM).
(**C**) Overlapping of proteins in B (TM and PTM) with those previously reported to be in the plasma membrane/cell wall.
For detailed information, refer to Table S2a. TM, transmembrane; Myr, myristoylation site; Pal, palmitoylation site; Pre, prenylation site; GPI, glycosylphosphatidylinositol anchor.

Functional categories of proteins

From the functional annotation of proteins in the current databases (National Centre for Biotechnology Information [NCBI], UniProtKB, The Institute for Genomic Research [TIGR], The Arabidopsis Information Resource [TAIR], Arabidopsis Membrane protein database [ARAMEMNON]) and the prediction by conserved functional domains, we were able to assign the 223 proteins into eight functional groups (**Figure 4**): signal transduction (38, 17.0%), transport (36, 16.1%), membrane trafficking and cellular organization (35, 15.7%), cell wall-related (13, 5.8%), stress response (19, 8.5%), protein destination and stability (20, 9.0%), metabolism (38, 17.0%), and protein–protein interaction (8, 3.6%). Those not clearly assigned to these eight groups were classified as "others" (16, 7.2%). Proteins involved in signal transduction, transport, membrane trafficking and metabolism are predominant in the pollen PM proteome.

The preferentially represented signal transduction group was characterized by calcium sensors, kinases and
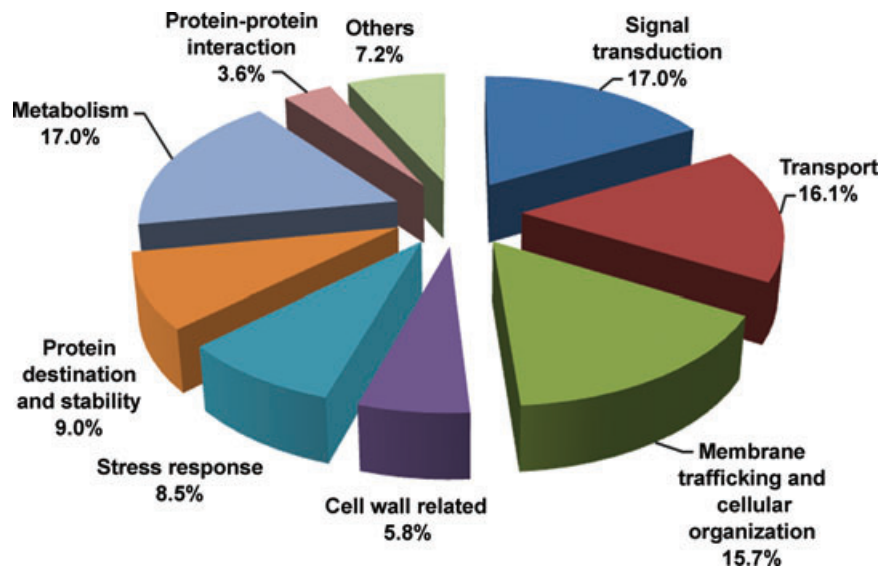
Figure 4. Functional classification of the 223 identified proteins in plasma membrane of lily pollen grains and pollen-tubes.

receptor-like kinases and small GTPases (**Table 1**). The membrane trafficking group included most components of the clathrin-dependent endocytosis pathway, whereas the transport group consisted of multiple transporters for protons and other solute such as sugars (**Table 1**). Our results highlight several novel proteins whose homologs had not been identified in previous PM proteomics studies of vegetative tissues and cultured cells (Table S2); examples are calcium-dependent protein kinase 2 (CDPK2 and 17), mitogen-activated protein kinase 7 (MAPK7), phosphatidylinositol 3-kinase (PI3K), small GTPases and transforming growth factor β receptor interacting protein, and gamma-adaptin and clathrin assembly proteins (Table S2). These findings are consistent with the function of polarity maintenance and growth of PTs (see Discussion section).

Quantitative difference between PG and PT PM proteins

Of the identified proteins, 65 could be quantified with at least three unique peptide MS/MS spectra and a P-value < 0.05 in the two independent experiments (Table S3, Figure S3). When tag 114 was set as the denominator, the ratios of 115:114 indicated variation between the two PT samples. According to the volcano plot shown in Figure S3, such variation is indistinct. The same applies to the ratios of 117:116 indicative of PG samples. Next, we examined proteins with different levels in PG and PT samples. Although most iTRAQ studies used a fold-change threshold of 1.2 or more for quantification (Duthie et al. 2007; Guo et al. 2007; Sui et al. 2008), we examined the data using F-test and Student's t-test to obtain statistically

significant quantitative information. The analysis revealed 14 of the 65 proteins with significant changes in levels between the two types of samples ($P < 0.05$) (**Table 2**). Figure S4 shows a representative MS/MS spectrum for a peptide derived from a clathrin heavy-chain protein. Among the 14 proteins with changed levels in PM during the transition from PGs to PTs, six were increased in level and include CDPK2, clathrin heavy chain, tubulin beta chain and tubulin alpha-1 chain proteins. In contrast, eight showed decreased levels and include PM ATPase 4, inorganic pyrophosphatase, and beta-adaptin (**Table 2**). These findings highlight the importance of calcium signaling and clathrin-dependent endocytosis in polarity maintenance and polarized growth of PTs (see Discussion section).

Discussion

Subcellular fractionation and prefractionation of peptides followed by LC-MS/MS represents a powerful system to analyze protein components of intracellular organelles as well as PM (Morel et al. 2006). In the present study, we used these techniques combined with iTRAQ labeling to identify pollen PM proteins and their quantitative differences in PGs and PTs. We identified 137 translation-related proteins in the pollen PM sample and 223 potential PM proteins. Proteins involved in signaling, membrane trafficking and transport were preferentially represented. Furthermore, our comparative analysis of PM samples from PGs and PTs revealed 14 proteins with differential expression in PGs and PTs.

Table 1. Identified proteins involved in signal transduction, transport and membrane trafficking

Accession	Protein annotations	Peptides (95%)	Conserved domain	kDa	pI	TM	PTM
Signal transduction (38)							
gi|7428011	Calcium-dependent protein kinase	1	S_TKc, EFh	59.4	5.93	–	Myr, Pal
gi|50080313	Calcium-dependent protein kinase 17	3	S_TKc, EFh	58.3	5.98	1	Myr, Pal
gi|77552937	Calcium-dependent protein kinase, isoform 2	3	S_TKc, EFh	59.4	5.9	1	Myr, Pal
gi|115465485	Mitogen-activated protein kinase 7	1	S_TKc	67	9.08	2	–
gi|77549870	Extracellular signal-regulated kinase 1	1	S_TKc	57	8.43	1	–
gi|50510074	Putative CRK1 protein	2	S_TKc	62.7	9.45	–	Myr, Pal
gi|37999984	Phosphatidylinositol 3-kinase-related protein kinase	0	PIKKc_SMG1	384	6.01	1	Myr
gi|79511817	Kinase	1	STYKc	78.6	8.88	3	Pal
gi|38345128	OsWAK receptor-like protein (OsWAK-RLP)	1	EGF_CA, STYKc	42.7	8.45	3	–
gi|53793399	Receptor-like protein kinase 1-like	1	LRRNT_2, LRR, STYKc	70.7	7.18	2	Myr, Pal
gi|15223445	Leucine-rich repeat transmembrane protein kinase	1	LRRNT_2, LRR_1, STYKc	65.4	7.46	2	Pal
gi|33146664	Receptor-like protein kinase	2	LRRNT_2, LRR, LRR_1, STYKc	69.8	7.62	1	Pal
gi|125556580	Phytosulfokine receptor precursor	1	DUF547 LRRNT_2, LRR, LRR_1	156.3	5.35	2	Pal
gi|125577549	Receptor-like protein kinase 2	1	LRRNT_2, LRR, LRR_1	74.9	6.35	1	Pal
gi|77552270	Leucine rich repeat family protein	1		96.5	9.05	–	Pal
gi|31433540	Nucleoside diphosphate kinase 1	1	NDK	16.8	6.84	–	–
gi|125546379	Phospholipase D	1	C2, PLDc, ZnF_C2HC	290	6.44	2	Pal
gi|125569828	ADP-ribosylation factor	3	ARF	25	8.51	1	Myr
gi|90101445	Rac-like GTP-binding protein 5	2	RHO	21.6	9.3	–	Pre, Pal
gi|7438422	GTP-binding protein rab2b	2	RAB	23.1	6.95	–	Pre, Pal
gi|46326983	Ethylene-responsive small GTP-binding protein	2	RAB	23.8	8.36	2	Pre, Pal
gi|125571145	Ras-related protein, putative	1	RAN	43.9	5.16	1	–
gi|77556132	GTP-binding protein SAR1A	1	SAR	22	6.91	–	–
gi|125605432	GTP-binding protein	1	YqeH	63.4	9.62	–	–
gi|50508716	Octicosapeptide/Phox/ Bem1p (PB1) domain-/ tetratricopeptide repeat (TPR)-containing protein	1	PB1	87.5	5.88	1	–
gi|30693337	Transducin family protein/WD-40 repeat family protein	2	WD40 domain	175.4	8.47	1	–

Table 1. Continued

Accession	Protein annotations	Peptides (95%)	Conserved domain	kDa	pI	TM	PTM
gi|42568419	Transducin family protein/WD-40 repeat family protein	1	WD40 domain, BROMO	186.9	6.66	1	Pal
gi|540535	Q group of receptor for activated C-kinase	1	WD40 domain	36.2	5.97	–	–
gi|54287644	Putative TGF-beta receptor interacting protein	1	WD40 domain	36.1	5.69	–	–
gi|42568981	SH3 domain-containing protein	2	SH3, Armadillo repeat	130.1	5.47	–	–
gi|108707344	SH3 domain containing protein	2	SH3, ARM repeat	115	5.21	–	Pal
gi|998430	GF14–6	4	14_3_3	29.7	4.76	–	–
gi|44917153	14–3-3 e-2 protein	4	14_3_3	29.4	4.72	–	–
gi|77555438	ABA-responsive protein	1	GRAM	31.6	6.31	1	–
gi|53792221	Putative non-phototropic hypocotyl 3	2	BTB/POZ domain, NPH3	70.6	5.46	1	–
gi|125591298	patched family protein	1	Patched	127.5	8.1	15	–
gi|125594819	Hypothetical protein (Calreticulin family protein)	1	Calreticulin	69.23	7.94	1	Pal
gi|125560038	Hypothetical protein (NPH3 family protein)	4	non-phototropic hypocotyl 3 (NPH3) and coiled-coil domains	93.09	5.2	1	Pal
Transport (36)							
gi|50838972	H-ATPase	18	Cation_ATPase_N, E1-E2_ATPase, ydrolase	105.4	6.36	10	–
gi|75232938	Plasma membrane ATPase (Proton pump)	16	Cation_ATPase_N, E1-E2_ATPase, Hydrolase	104.8	6.34	10	Pal
gi|584794	Plasma membrane ATPase 1 (Proton pump 1)	13	Cation_ATPase_N, E1-E2_ATPase, Hydrolase	105.2	6.43	10	–
gi|124360090	Plasma-membrane proton-efflux P-type ATPase	14	Cation_ATPase_N, E1-E2_ATPase, Hydrolase	105.6	6.51	10	–
gi|47497038	Putative H+-exporting ATPase	12	Cation_ATPase_N, E1-E2_ATPase, Hydrolase	104.2	6.05	10	–
gi|416664	Plasma membrane ATPase 4 (Proton pump 4)	14	Cation_ATPase_N, E1-E2_ATPase, Hydrolase	105.2	6.52	10	–
gi|15229126	Arbbidopsis H(+)-ATPase 8 (AHA8)	13	Cation_ATPase_N, E1-E2_ATPase, Hydrolase	104.1	5.53	10	–
gi|55274626	Plasma membrane proton ATPase 5	9	Cation_ATPase_N, E1-E2_ATPase, Hydrolase	102.1	5.56	10	–
gi|64460298	Proton P-ATPase	12	Cation_ATPase_N, E1-E2_ATPase, Hydrolase	104.6	6.03	10	–
gi|5669151	Plasma membrane proton ATPase	11	Cation_ATPase_N, E1-E2_ATPase, Hydrolase	104.8	6.48	10	–
gi|125534415	Plasma membrane ATPase	1	E1-E2_ATPase	113.2	9.05	14	Pal
gi|125586110	Phospholipid-transporting ATPase 1	1	E1-E2_ATPase	76.5	6.66	3	Pal
gi|125526179	PDR-type ABC transporter	2	ABC2_membrane, PDR_assoc, AAA	174	8.99	11	Pal
gi|75330898	Pleiotropic drug resistance protein 13	4	ABC2_membrane, PDR_assoc, AAA	161.6	7.59	14	–

Table 1. Continued

Accession	Protein annotations	Peptides (95%)	Conserved domain	kDa	pI	TM	PTM
gi|42569461	Pleiotropic drug resistance 3	3	ABC2_membrane, PDR_assoc, AAA	159.9	8.74	13	–
gi|75322003	Pleiotropic drug resistance protein 12	1	ABC2_membrane, PDR_assoc, AAA	169.1	8.75	14	Pal
gi|15231301	RNase L inhibitor protein 1 (ABC transporter E family member 1)	2	RLI, Fer4, AAA	68.2	8.43	–	Pal
gi|125577451	Hypothetical protein (similar to ATP-binding cassette sub-family E member 1)	3	RLI, Fer4, AAA	70.9	9.05	–	Pal
gi|15222849	Xanthine/uracil permease family protein	1	Xan_ur_permease	57.8	9.2	12	Pal
gi|15231001	Sugar transporter	1	Sugar_tr, MFS_1	56.2	8.35	11	–
gi|30683925	Vacuolar proton ATPase A1 (VHA-A1)	2	V_ATPase_I	93.4	5.98	6	–
gi|125528283	Vacuolar ATP synthase 98 kDa subunit	2	V_ATPase_I	96	5.93	7	Myr, Pal
gi|15226542	Vacuolar proton ATPase A2	1	V_ATPase_I	93.1	5.38	6	Pal
gi|125595424	Similar to putative vacuolar ATP synthase subunit C	1	V-ATPase_C	96.7	6.68	–	–
gi|124360985	H^+-transporting two-sector ATPase, C (AC39) subunit	2	vATP-synt_AC39	40.8	4.94	–	–
gi|57863812	Putative YLP (vacuolar ATP synthase subunit E)	1	vATP-synt_E	26	9.37	–	–
gi|857574	H^+-ATPase	1	ATP-synt_C	16.7	8.62	4	–
gi|1352830	Vacuolar ATP synthase catalytic subunit A	7	ATP-synt_ab, ATP-synt_ab_C	62	5.88	–	–
gi|53793338	Vacuolar ATPase B subunit	5	HAS-barrel, ATP-synt_ab_N, ATP-synt_ab, ATP-synt_ab_C	54.1	5.07	–	–
gi|50251203	Putative vacuolar proton-ATPase	7	ATP-synt_ab_N, ATP-synt_ab, ATP-synt_ab_C	68.6	5.37	–	–
gi|111162629	ATP-synthase	2	P-loop NTPase	18.7	5.15	–	–
gi|42407865	Putative mitochondrial F0 ATP synthase D chain	1	ATP-synt_D	19.7	5.19	–	–
gi|19685	ATP synthase beta subunit	6	ATP-synt_ab_N, AAA	59.9	5.95	1	–
gi|50509197	Putative ATP synthase delta chain	2	ATP-synt_DE_N	21.2	5.72	–	GPI
gi|125593659	Similar to ADP, ATP carrier protein	3	Mito_carr	41	9.75	6	–
gi|790479	Inorganic pyrophosphatase	6	H_Ppase	80.2	5.03	16	Pal
Membrane trafficking and cellular organization (35)							
gi|125578212	Clathrin heavy chain	135	Clathrin_propel, Clathrin-link, CLH	193.4	5.25	–	–
gi|42563757	Clathrin heavy chain	87	Clathrin_propel, Clathrin-link, CLH	193.3	5.25	–	–
gi|30681617	Clathrin heavy chain	90	Clathrin_propel, Clathrin-link, CLH	193.2	5.26	–	–

Table 1. Continued

Accession	Protein annotations	Peptides (95%)	Conserved domain	kDa	pI	TM	PTM
gi|15218697	Epsin N-terminal homology (ENTH) domain-containing protein/clathrin assembly protein-related	3	ENTH	67	4.84	–	Myr
gi|18379261	Epsin N-terminal homology (ENTH) domain-containing protein	3	ENTH	63.6	5.11	–	Myr
gi|30679231	Epsin N-terminal homology (ENTH) domain-containing protein/clathrin assembly protein-related	3	ENTH	68.2	5.22	–	–
gi|15242060	Epsin N-terminal homology (ENTH) domain-containing protein/clathrin assembly protein-related	2	ENTH	66.6	4.99	2	Myr
gi|125543307	Clathrin assembly protein	1	ENTH	72	5.44	1	–
gi|50251206	Synaptosomal-associated protein 91-like	0	ENTH	66.1	5.6	2	Myr, Pal
gi|55296045	Putative gamma-adaptin 1	2	Adaptin_N, Alpha_adaptinC2	95.1	5.81	–	–
gi|15236506	Beta-adaptin, putative	9	Adaptin_N, HEAT, Alpha_adaptinC2, B2-adapt-app_C	99.1	4.91	–	–
gi|15219810	Clathrin adaptor complexes medium subunit family protein	2	Adap_comp_sub	49	6.01	–	GPI
gi|30688616	Gamma-adaptin 1	1	Alpha_adaptinC2	96.5	5.46	–	Pal
gi|90398981	Similar to clathrin light chain	9	Clathrin_lg_ch	32.5	4.96	–	–
gi|18409442	Similar to protein transporter/clathrin light chain	3		27	5.94	–	–
gi|125556549	Similar to EH domain-containing protein 1	3	EFh, Dynamin_N	61.1	6.75	–	–
gi|38345297	EH domain-containing protein 1	2	EFh, Dynamin_N	61	7.2	–	–
gi|125595166	Dynamin family protein	3	DYNc, GED	74.3	9.48	1	–
gi|125533006	Dynamin family protein	1	DYNc, GED	79.6	6.39	–	–
gi|52076761	Dynamin	1	DYNc, Dynamin_M, PH, GED	99.2	9.21	–	–
gi|18418491	SH3 domain-containing protein2	1	SH3	40.9	6.44	–	–
gi|78708906	SH3 domain-containing protein3	1	SH3	41.2	6.95	–	–
gi|125533174	Vacuolar-sorting receptor precursor	0	PA, EGF_like, Bac_surface_Ag	142.7	7.79	4	Pal
gi|18417560	Integral membrane Yip1 family protein	1	Yip1	30.2	7.74	5	GPI
gi|77554057	Exocyst complex component Sec10	1	Sec10	88.2	5.12	–	–
gi|20804648	Vesicle transport v-SNARE protein-like	1	V-SNARE	24.9	9.13	1	Pal
gi|125554666	Kinesin motor protein-related)	2	CH, KISc	99.3	5.87	1	–

Table 1. Continued

Accession	Protein annotations	Peptides (95%)	Conserved domain	kDa	pI	TM	PTM
gi|15221848	Myosin-like protein XIE; motor/protein binding	1	Myosin_N, MYSc, IQ, DIL	173.6	9.11	2	Pal
gi|30678420	TPLATE; binding	1	–	130.9	5.67	1	
gi|77548871	Actin-7	17	ACTIN	41.6	5.31	–	Pal
gi|50058115	Actin	15	ACTIN	41.6	5.31	–	Pal
gi|1498384	Actin	10	ACTIN	37.1	5.46	–	–
gi|3219763	Actin-53	8	ACTIN	37.4	5.39	–	–
gi|49387751	Tubulin beta chain	6	Tubulin, Tubulin_C	50.1	4.72	–	Pal
gi|50509129	Tubulin alpha-1 chain	5	Tubulin, Tubulin_C	49.7	4.92	–	–

The protein functions were assigned according to the BLAST and motif searches. Myr, myristoylation ; Pal, palmitoylation; Pre, prenylation ; PTM, posttranslational modification ; TM, transmembrane domain. Details are in Table S2.

Table 2. Differentially expressed proteins in plasma membrane of lily pollen grains and pollen tubes

Accession	Protein name	% Cov	Pep.	P-value F-test	P-value t-test	Mean (PT/PG) $\pm$ SD
Signal transduction						
gi|77552937	Calcium-dependent protein kinase, isoform 2	16.5	3	0.101 928	0.027 025	1.983 $\pm$ 0.032
Transport						
gi|416664	Plasma membrane ATPase 4	52.6	14	0.384 576	0.018 213	0.760 $\pm$ 0.012
gi|790479	Inorganic pyrophosphatase	17.1	6	0.278 875	0.019 054	0.642 $\pm$ 0.011
gi|19685	ATP synthase beta subunit	41.4	6	0.387 916	0.009 05	0.504 $\pm$ 0.011
Membrane trafficking						
gi|49387751	Tubulin beta chain	37.8	6	0.972 98	0.035 306	1.645 $\pm$ 0.227
gi|50509129	Tubulin alpha-1 chain	26.2	5	0.154 51	0.037 929	1.300 $\pm$ 0.017
gi|125578212	Clathrin heavy chain	82.7	135	0.060 393	0.016 024	1.121 $\pm$ 0.001
gi|15236506	Beta-adaptin	21.5	9	0.113 238	0.008 201	0.679 $\pm$ 0.002
Metabolism						
gi|15623805	ATP citrate lyase a-subunit	35.2	9	0.337 12	0.010 224	0.556 $\pm$ 0.008
Protein synthesis						
gi|125581425	Ribosomal protein L5	41.9	5	0.058 128	0.015 186	1.801 $\pm$ 0.010
gi|50509811	Ribosomal protein L18	23.6	4	0.180 301	0.016 584	1.734 $\pm$ 0.029
gi|12246885	Ribosomal protein S10	43.7	3	0.265 886	0.043 785	0.725 $\pm$ 0.010
gi|4038471	40S ribosomal protein S27 homolog	45.3	3	0.639 472	0.045 476	0.593 $\pm$ 0.045
Other						
gi|35215184	Polyadenylate-binding protein	30.6	6	0.993 891	0.030 937	0.624 $\pm$ 0.049

The differentially expressed proteins assessed by F-test and Student's t-test are listed with their P-values. % Cov, percentage of amino acids matching the identified peptides with confidence > 0 divided by the total number of amino acids in the sequence. Pep., the number of peptides dectected with confidence $\geq$95%. Average (PT/PG) (pollen tube/pollen grain) $\pm$ SD shows the fold change of the differentially expressed proteins. Detailed information is in Table S1 and Table S3. ATP, adenosine tri-phosphate.

Importance of translational apparatus proteins identified in the pollen PM

Similar to other PM proteomics findings (Alexandersson et al. 2004; Marmagne et al. 2004; Natera et al. 2008), we identified translational-apparatus and DNA/RNA-binding proteins with preferential numbers of ribosome proteins in the pollen PM samples, although immunoblotting analysis of marker proteins for cytoplasm and other organelles clearly showed that the purified PM vesicles were highly enriched, with little contamination

from cytoplasm and other organelles (**Figure 2**). Several studies have reported or argued for the existence of the "PM-bound ribosomes" in animal cells (Baker 1967; Grasso et al. 1978; Spacek 1982). Recent proteomics studies of yeast and higher plants revealed a relatively high proportion of ribosomal and other proteins of translational apparatus in PMs (Navarre et al. 2002; Alexandersson et al. 2004) and in detergent-resistant PM (DRM) (Lefebvre et al. 2007). For instance, ribosomal proteins accounted for 50% of the identified PM proteins in budding yeast (Navarre et al. 2002), 21.7% of the identified leaf PM proteins of *Arabidopsis* (Alexandersson et al. 2004), and 18.5% of the identified root DRM proteins of *Medicago truncatula* (Lefebvre et al. 2007). The identification of these proteins in different PM proteomes probably resulted from cytoskeleton-binding polysomes anchored to the PM via actin filaments (Davies et al. 1991; Hesketh 1996). Recent study of lipid rafts by immunoblotting and immunofluorescence analyses showed that some ribosomal proteins were targeted to the raft via S-acylation, which provides direct evidence for localization of ribosome proteins in defined domains of PM (Yang et al. 2009).

Polarized growth of PTs exhibits the following unique features: (i) the growth is confined to a tip PM domain to which membrane materials transport and fuse; (ii) highly active metabolic/cellular processes are focused in the apical region; (iii) actin cytoskeleton, along with the PT and attached to the PM by actin filaments, is crucial for maintaining the morphological feature of the tube (Cheung and Wu 2008); and (iv) comet-like F-actin in apical cytosol and actin patches at the PM, similar to those in polarized domain in animal cells, are required for polarity maintenance and/or polarized cell growth (Cheung and Wu 2008; Nakayama et al. 2009). Consistent with these features, a recent study revealed the colocalization of cytoskeleton and cytoskeleton-associated large ribonucleoprotein particles, which were identified to contain translational-apparatus proteins, such as ribosomal proteins, eIFs and EFs, DNA/RNA-binding proteins, and a set of storage mRNA in tobacco and *Arabidopsis* pollen (Honys et al. 2009). These observations suggested that such organization was devoted to regulation of protein synthesis, processing and precise localization and was useful in fast tip-growing pollen tubes (Honys et al. 2009). All lines of evidence support our identification of translation-related proteins in pollen PM samples. Therefore, such proteins may localize nearby or associate with the PM. Furthermore, our data showed four ribosome proteins with changed levels during the developmental transition from PGs to PTs in the PM sample. Together, these data suggest that the translational-apparatus proteins combined with DNA/RNA-binding proteins organized nearby or associated with the PM, possibly with apical PM, may represent a special mechanism to facilitate protein synthesis of nearby polarized growth domains for fast growth of PTs. The detailed functional characterization of the translational apparatus combined with DNA/RNA-binding proteins awaits further investigation.

Pollen PM proteome appears specialized for the polar growth of pollen tubes

Cell polarity and membrane domain formation are essential for cell differentiation and development (Zappel and Panstruga 2008). The PT is a specialized polarized cell type with a defined, specialized domain at the apical PM to facilitate the polarized growth of the tube. Our data showed that the pollen PM proteome preferentially contained proteins related to signaling, membrane trafficking and ion transportation, which is comparable to PM proteomes identified from vegetative tissues and cultured cells in plants (Alexandersson et al. 2004; Marmagne et al. 2004, 2007; Morel et al. 2006). Indeed, the relative high proportion of proteins (53.4%) we identified are homologs identified previously in the PM proteomes of vegetative tissues and cultured cells (**Figure 3**, Table S2). However, compared with PM proteomes from other types of cells, the pollen PM from lily appeared to be enriched in small GTPases, kinases, endocytosis-related proteins and ion transporters. Several proteins without corresponding homologs reported in PM proteomes of other types of plant cells include CDPK2 and 17, MAPK7, PI3K, transforming growth factor β receptor interacting protein, and gamma-adaptin and clathrin assembly proteins (Table S2).

The identified small GTPase proteins include Rho/Rac (named Rop/Rac in plants), Rab, Arf/Sar, and Ran (**Table 1**) from four small GTPase subfamilies known in plants (Vernoud et al. 2003). Studies have revealed the central roles of tip PM-localized ROPs in polarized growth of PTs (Cole and Fowler 2006). Rabs identified in *Arabidopsis* and tobacco PTs are involved in polarized membrane trafficking (de Graaf et al. 2005; Szumlanski and Nielsen 2009). In mammalian cells, Arf/Sar functions mainly in the endosomal-PM system, where it is involved in recycling to the PM, regulated secretion, and coordinating the actin cytoskeleton (Holstein 2002). Ran plays a role in controlling nucleoprotein transport and microtubulin organization (Dallol et al. 2009). The function of small GTPases requires an interconnection with other signal molecules (Cole and Fowler 2006). Studies of *Petunia* PiCDPK and tomato receptor-like kinase LePRK showed the two proteins participating in polarity signaling, possibly via affecting ROP activity (Cole and Fowler 2006; Yoon et al. 2006), but the roles of calcium sensors and kinases in the signaling cascade required for polarized growth of PTs are still unclear (Yang 2008). Our identification revealed a relatively large number of pollen PM-localized kinase proteins, including the calcium sensors CDPKs, MAPK, cdc2-related kinase, extracellular signal-regulated kinase, PI3K and receptor-like kinases (**Table 1**), and CDPK2 level increases significantly in PTs. These findings

provide extensive information of signal molecules localized in pollen PM for further understanding their interactions in directing polar growth of PTs.

One of most important cellular features of PT cells is polarized tip growth, which depends on actively targeting and fusing vesicles by polarized exocytosis motored by actin filaments. The polarized exocytosis transports membrane and wall materials to the defined domain for the newly synthesized plasma membrane and cell wall (Cole and Fowler 2006). Cytological studies suggested that excess vesicles, estimated to be 79% in tobacco PTs (Ketelaar et al. 2008), are endocytically internalized at the expanding apex (Cheung and Wu 2008). Our study revealed diverse components of a clathrin-dependent endocytosis pathway in the PM fraction; examples are clathrin heavy and light proteins, clathrin assembly proteins, adaptin, dynamin, Src homology 3 domain-containing proteins, motor proteins, Soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins and the aforementioned Raf/Sar and Rab GTPases (**Table 1**), which cover most of the proteins involved in the pathway (Holstein 2002; Murphy et al. 2005). The identified clathrin and adaptin displayed significant changes in levels in the PM during the developmental transition from PG to PT growth (**Table 2**). This finding indicates that at protein levels, clathrin-dependent endocytosis is an important pathway of endocytosis in PTs, and active endocytosis may be crucial for polarized growth of PTs.

Although no direct molecular evidence defines the precise roles of endocytosis in polarized PT growth, endocytosis balanced with diffusion and direct transport is sufficient for polarized protein distribution in animal cells (Marco et al. 2007). For example, dynamin plays a key role in maintaining polarity within defined regions of the PM in the early embryo of *Caenorhabditis elegans* by regulating the polarity signal molecule Rho GTPase to a defined PM domain (Nakayama et al. 2009). Cytological observations showed that endocytic activity predominantly occurs at the subapical PM of PTs (Cheung and Wu 2008). These lines of evidence suggest that endocytosis may represent an important mechanism regulating polarized localization of proteins and maintenance of a defined PM domain. In addition, the polarized exocystosis to a region of the PM of animal cells experiencing rapid polarized growth is required for tightly controlled delivery of specific membrane and protein components (Hsu et al. 2004). PT growth oscillates between fast and slow growth phases: ROP1 is initially activated at the center region of the future apical cap, leading to fast polarized growth; then it spreads laterally to the subapical region, thereby slowing down the polarized growth (Yang 2008). Therefore, our data, combined with these observations, suggest a possible model that the exocytosis-mediated fast growth phase of PTs results in lateral spreading of tip PM-localized signal molecules such as ROPs, whereas endocytosis occurring in the subapical region recycles the signal molecules spreading laterally to the region.

The interconnection of the two membrane trafficking pathways tightly tethers the molecules in the apical PM. Further studies by combined genetic and cytological approaches are required to dissect the links between endocytotic activity and tip PM localization of signal molecules.

In summary, we identified the protein components of pollen PM and quantitatively determined differences in protein levels between the PG and PT PM. Our study revealed novel features of PG and PT PM and highlighted several novel PM proteins related to polarized PT growth. Importantly, the study suggested that clathrin-dependent endocytosis might be an important endocytosis pathway in PTs, which may be crucial for polarized growth of PTs. These data shed light into the mechanism underlying polarized PT growth.

Materials and Methods

Mature pollen and *in vitro* germination

Mature PGs were collected from *Lilium davidii* Duch. planted under the natural growing season in Gansu Province, China. The PGs were dried briefly at room temperature and then kept at -80 °C. *In vitro* germination was performed as described (Ren et al. 1998; Prado et al. 2004) with several modifications. Briefly, the PGs were first transferred to -20 °C for 2 weeks before the germination experiments and then hydrated at 4 °C for 10 h in a dark humid environment to induce self-healing and washed twice with an isotonic buffer to remove the surrounding oily and pigmented substance. Thereafter, the pretreated PGs were directly used for preparation of plasma membrane or transferred to a liquid germination medium (1.6 mM H_3BO_3, 1.0 mM KCl, 500 μM $CaCl_2$ and 15% [w/v] sucrose, pH 6.0). PGs in the germination medium were cultured in the dark at 28 °C for 2 h with gentle shaking at 75 r/min. This procedure gave rise to a germination rate of more than 90% with PTs of approximately 500 μm long. PTs were collected by using cell strainers (100 μm) and were centrifuged at 1 000 × *g* for 5 min.

Preparation of plasma membrane from PGs and PTs

Pollen grains and PTs were homogenized in a homogenization medium (15% [w/v] sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM ascorbic acid, 0.6% [w/v] polyvinylpolypyrrolidone, 1 mM dithiothreitol (DTT), 1 mM phenylmethanesulfonyl fluoride, protease inhibitor cocktail and 50 mM MOPs/KOH, pH 7.8) with FastPrep-24 (MP Biomedicals, Irvine, CA, USA). After differential centrifugation, PM-containing microsomes were collected after centrifuging at 100 000 × *g* at 4 °C for 1 h. The PM vesicles were purified by use of an aqueous polymer two-phase partitioning system, one of the most efficient methods used to isolate PM vesicles from animal and plant materials (Larsson et al. 1987; Morre

and Morre 2000; Marmagne et al. 2007; Kierszniowska et al. 2009). The composition of the partitioning system used here is 6.3% (w/w) Dextran T-500, 6.3% (w/w) polyethylene glycol (PEG) 3350, 250 mM sucrose, 5 mM KCl, 1 mM DTT, and 5 mM K-phosphate, pH 7.8. After partitioning, the collected upper phase was diluted fourfold with a dilution buffer (250 mM sucrose, 1 mM DTT, 1 mM phenylmethanesulfonyl fluoride, protease inhibitor cocktail and 50 mM MOPs/KOH, pH 7.8), and then centrifuged at 200 000 $\times$ g at 4 °C for 1 h to collect PM vesicles. The PM vesicles were washed by resuspending in the dilution buffer and then pelleted by centrifugation.

The resulting PM vesicles were further washed with a Brij58- and KCl-containing buffer (0.05% [w/v] Brij58, 0.2 M KCl, 250 mM sucrose, protease inhibitor cocktail and 5 mM K-phosphate, pH 7.8) at a detergent-to-protein ratio of 10:1 in micrograms as previously described (Johansson et al. 1995; Alexandersson et al. 2004). After centrifugation at 200 000 $\times$ g at 4 °C for 1 h, the purified PM vesicles were resuspended in the dilution described above and stored at −80 °C. Protein concentration was determined by the Bradford assay (Bradford 1976) with bovine serum albumin used as a standard.

Purity analysis of PM vesicles

Proteins were separated on a 12.5% sodium dodecyl sulfate (SDS) polyacrylamide gel and then electrotransferred onto a polyvinylidene difluoride membrane (Pierce, IL, USA) with 10 mM 3-cyclohexylamino-1-propanesulfonic acid and 10% (v/v) methanol at a constant current of 2 mA per cm^2 of gel area for 1 h as previously described (Dai et al. 2006). Primary antibodies used were against PM P-type ATPase (VHA) from *V. faba* (Kinoshita and Shimazaki 1999), OsRad21– 3 from *Oryza sativa* (Tao et al. 2007), mitochondrial cytochrome oxidase subunit II (COXII; from *Arabidopsis*, Agrisera no. AS04 053A, Stockholm, Sweden), V-ATPase (Agrisera no. AS07 213, Stockholm, Sweden), peroxisome catalase from *Cucurbita sp.* Amakuri Nankin (Yamaguchi et al. 1984), and cFBPase (cytosolic fructose 1,6 biphosphatase from *Arabidopsis*, Agrisera no. AS04 043, Stockholm, Sweden). All antibodies were used at 1:4 000 dilution, except OsRad21-3 at a dilution of 1:2 000.

Protein digestion, iTRAQ labeling and strong cation-exchange fractionation

Proteins (114 μg) from purified PM vesicles were precipitated in cold acetone overnight, collected by centrifugation at 20 000 $\times$ g at 4 °C for 30 min, and dissolved in 1% SDS, 100 mM triethylammonium bicarbonate, pH 8.5. After reduction and alkylation and trypsin digestion (Zhu et al. 2009), peptides were labeled by the use of the iTRAQ four-plex kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions, with iTRAQ 114 and 115 for the

PT samples and 116 and 117 for the PG sample. Labeled PT and PG samples were mixed and lyophilized. For strong cation-exchange (SCX), the peptide mixtures were loaded in solvent A (25% [v/v] acetonitrile, 10 mM ammonium formate, pH 2.8) onto a polysulfoethyl A column (2.1 $\times$ 100 mm, 5 μm, 300 Å; PolyLC, Columbia, MD, USA) and separated through a 0–20% linear gradient of solvent B (25% [v/v] acetonitrile, 500 mM ammonium formate) for 50 min, and then 100% solvent B for 15 (Agilent high performance liquid chromatography [HPLC] system 1100). The flow rate was 200 μL/min. For each sample, two independent biological replicates were used.

Reverse-phase nanoflow LC-MS/MS and data analysis

Each SCX fraction was lyophilized and dissolved in solvent A containing 3% [v/v] acetonitrile, 0.1% [v/v] acetic acid and 0.01% (v/v) trifluoric acetic acid. Peptides were loaded onto a C18 capillary trap cartridge (LC Packings, Amsterdam, Netherlands) and then separated on a 15 cm nanoflow C18 column (PepMap 75 μm diameter, 3 μmm, 100 A) (LC Packings) by a linear gradient from 3% to 40% of solvent B (96.9% [v/v] acetonitrile and 0.1% [v/v] acetic acid) for 2 h, then ramped to 90% solvent B for 10 min at a flow rate of 200 nL/min. The separated peptides were sprayed into the orifice of a quadruple time-of-flight mass spectrometer QSTAR XL (Applied Biosystems), which was operated in an information-dependent data acquisition mode with one survey scan followed by three product ion scans of the three most abundant peptides acquired in each cycle (Zhu et al. 2009). The mass measurement accuracy for both the precursor and product ions is within 10 ppm.

The MS/MS data were submitted to database search considering biological modification and amino acid substitution against the NCBI nonredundant database (5 222 402 entries, downloaded on 2 July 2007) by use of the Paragon algorithm of ProteinPilot v2.0.1 (Applied Biosystems). Plant species, fixed modification of methylmethanethiosulfate labeled cysteine, fixed iTRAQ modification of free amine in the N-terminus and lysine, and variable iTRAQ modifications of tyrosine were considered. Parameters such as trypsin digestion, precursor mass accuracy and fragment ion mass accuracy are built-in settings of the software. The raw peptide identification results from the Paragon algorithm were further processed by the ProGroup algorithm. The software calculates a percentage confidence that reflects the probability that the hit is a false positive. In the iTRAQ experiments, at least one highly confident MS/MS spectrum is needed for identification. For proteins with one significant contributing peptide, the MS/MS spectrum of the peptide was manually inspected. The false discovery level was estimated by performing the search against a concatenated database containing both forward and reverse sequences.

For relative protein quantification, only MS/MS spectra unique to a particular protein and a signal-to-noise ratio of

> 9 for all iTRAQ were used for quantification (software default settings, Applied Biosystems). The mean, standard deviation, and *P*-values to estimate statistical significance of the protein changes were calculated. A differentially expressed protein needs to have at least three MS/MS spectra (allowing generation of a *P*-value) and a *P*-value < 0.05. Variations between replicates (PT/PT, 115:114 and PG/PG 117:116) and the two types of samples (PG/PT, 116:114 and 117:115) were assessed by volcano plot, and the significance of differential expression was determined by *F*-test and Student's *t*-test.

In silico analysis

Molecular features, including mass (dalton) and isoelectric point (pI) of identified proteins, were automatically calculated with the ProtParam tool on the ExPasy server (http://www.expasy.ch/tools/protparam.html). Conserved domains were detected by the SMART program (http://smart.embl-heidelberg.de/). Transmembrane (TM) domains were predicted by HMMTOP v.2.0 (http://www.enzim.hu/hmmtop/). Posttranslational modification results (myristoylation, prenylation, glycosylphosphatidylinositol anchor and palmitoylation) were predicted by the use of the following tools: myristoylation with N-myristoyltransferase (NMT) predictor (http://mendel.imp.ac.at/myristate/SUPLpredictor.htm), PlantsP (http://plantsp.genomics.purdue.edu/plantsp/html/myrist.html), Myristoylator (http://www.expasy.ch/tools/myristoylator/) and TermiNator (http://www.isv.cnrs-gif.fr/terminator2/index.html); glycosylphosphatidylinositol anchor with big-PI Plant Predictor (http://mendel.imp.ac.at/sat/gpi/plant_server.html), and GPI-SOM (http://gpi.unibe.ch/); prenylation with PrePS (http://mendel.imp.ac.at/sat/PrePS/index.html); and Palmitoylation with CSS-Palm 2.0 and TermiNator. Signal peptides were predicted with SignalP (http://www.cbs.dtu.dk/services/SignalP/). Protein annotations were collected from NCBI (http://www.ncbi.nlm.nih.gov/), UniProt (http://www.uniprot.org/), ARAMEMNON (http://aramemnon.botanik.uni-koeln.de/), TIGR (http://www.tigr.org/db.shtml) and TAIR (http://www.arabidopsis.org/).

Acknowledgements

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Cation exchange chromatography of isobaric tags for relative and absolute quantitation (iTRAQ)-labeled peptides derived from lily pollen grain and pollen tube plasma membrane proteins.

Figure S2. Detailed tandem mass spectrometry information of proteins identified by Paragon algorithm with only one significant contributing peptide.

Figure S3. Volcano plot representation of isobaric tags for relative and absolute quantification (iTRAQ) results.

Figure S4. A representative MS/MS spectrum showing protein identification and relative quantification of a clathrin heavy chain protein in the plasma membrane samples of pollen grains and pollen tubes.

Table S1. Raw data for protein identification and quantification.

Table S2. Annotation and classification of identified proteins.

Table S3. Identified proteins with usable quantification results.

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