

Free-flow electrophoresis of plasma membrane vesicles enriched by two-phase partitioning enhances the quality of the proteome from *Arabidopsis* seedlings

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Abstract

The plant plasma membrane is the interface between the cell and its environment undertaking a range of important functions related to transport, signaling, cell wall biosynthesis and secretion.

Multiple proteomic studies have attempted to capture the diversity of proteins in the plasma membrane using biochemical fractionation techniques. In this study, two-phase partitioning was combined with free-flow electrophoresis to produce a population of highly purified plasma membrane vesicles that were subsequently characterized by tandem mass spectroscopy. This combined high-quality plasma membrane isolation technique produced a reproducible proteomic library of over 1,000 proteins with an extended dynamic range, including plasma membrane-associated proteins. The approach enabled the detection of a number of putative plasma membrane proteins not previously identified by other studies, including peripheral membrane proteins. Utilizing multiple data sources, we developed a PM-confidence score to provide a value indicating association to the plasma membrane. This study highlights over 700 proteins that, while seemingly abundant at the plasma membrane, are mostly unstudied. To validate this dataset, we selected 14 candidates and transiently localized 13 to the plasma membrane using a fluorescent tag. Given the importance of the plasma membrane, this dataset provides a valuable tool to further investigate important proteins. The mass spectrometry data are available via ProteomeXchange, identifier PXD001795.

Keywords

Plasma membrane, *Arabidopsis*, free-flow electrophoresis

Introduction

The plasma membrane (PM) physically defines the boundary between each cell and its environment. Aside from being a physical barrier, the PM is also the interface for the exchange of nutrients and metabolites, and is the primary site for extracellular signal perception. As such, the PM is a dynamic structure, ready to respond to stimuli by triggering downstream signaling events. Active vesicle transport to and from cytoplasmic organelles allows a rapid modification of PM composition and cell wall deposition. The PM is highly heterogeneous, due to the variety of tasks it needs to accomplish¹. Furthermore, the composition and quantity of PM proteins varies with cell type, developmental state, and environment, with each cell containing potentially unique combinations of different protein classes such as transporters, receptors, and signaling components.

While about 30 % of genes are predicted to encode membrane proteins in eukaryotes^{2,3}, these are often underrepresented in whole-tissue shotgun proteomic surveys. Among these membrane-associated proteins, only a fraction are delivered to the PM. However, unlike other compartments, the signals and mechanisms that target proteins to the PM remain unclear. Recently, an LxxxA motif was identified as necessary to target a subfamily of aquaporins to the PM in maize. However, this signal is not sufficient to target other aquaporins to the PM and the targeting mechanism seems unclear since this motif is found within a transmembrane domain of the protein, making direct interaction with vesicle coat proteins unlikely⁴. Given the unknown elements surrounding protein targeting to this key cellular compartment, identification of PM proteins requires high quality isolation procedures and proteomic analysis. Furthermore,

characterization of integral proteins poses some technical challenges due to their hydrophobicity, heterogeneity, and low relative abundance⁵.

To analyze the PM proteome, it is necessary to first isolate microsomal fractions, followed by the enrichment of PM vesicles by differential centrifugation⁶. PM enrichment can be achieved by a variety of techniques including density gradient centrifugation⁷, free-flow electrophoresis⁸, or more commonly by two-phase partitioning (2PP)^{9, 10}. In 2PP, outside-out orientated plasma membrane vesicles remain in the upper phase of a polyethylene glycol / dextran mixture during centrifugation, resulting in a sufficiently pure preparation for proteomic profiling by LC-MS/MS. Purity can be enhanced by washing the upper phase contents in ammonium bicarbonate or detergents¹¹⁻¹⁴, though this may compromise detection of loosely-associated peripheral proteins, which are of functional importance at the plasma membrane. Repeated rounds of two-phase partitioning also increases purity but inevitably decreases the final yield, since only about 70 % of PM vesicles are retained in the upper phase¹⁵. The highest purity plasma membrane fractions have come from cell suspension cultures, especially where gentle homogenization methods such as nitrogen decompression^{14, 16} or Potter-Elvehjem homogenization¹¹ were used. To date, over 1500 unique proteins have been identified in cell suspension culture plasma membrane proteomes, with the most common contaminants being vacuolar, ER and cytosolic proteins^{11, 13, 17-19}. In addition to these membrane contaminants, highly abundant photosystem proteins, particularly plastid proteins (*e.g.* ribulose-1,5-bisphosphate carboxylase/oxygenase), are problematic when using autotrophic systems such as liquid- or soil-grown plants^{12, 20}. Thus far, nearly 4000 proteins from 30 separate studies have been assigned to the PM proteome from the reference plant *Arabidopsis* using various experimental systems²¹. Increasing the bank of plasma

membrane profiles from plant and liquid-grown plantlets will mean genuine plasma membrane proteins, particularly peripheral proteins, are repeatedly identified and can be localized to the plasma membrane with confidence. In an effort to increase the diversity of proteins assigned to the PM, we enriched vesicles from Arabidopsis seedlings and employed a combination of 2PP and free-flow electrophoresis (FFE) with tandem mass spectrometry to profile this important membrane system. The approach has redundantly identified 1029 proteins, with about 70 % associated with the PM. Significantly, compared to other techniques our approach was able to capture peripheral and soluble proteins associated to the PM.

Experimental Section

Plant material and growth

For the characterization of the plasma membrane by FFE, 50 mg batches of seeds from *Arabidopsis thaliana* (L.) Heynh. accession Columbia (Col-0) were surface-sterilized by rinsing in 96 % ethanol for 1 min, followed by 5 min rinse with a sterilization solution (50 % household bleach, 0.1 % Tween-20) and five washes with sterile distilled water, under a sterile hood.

Imbibed seeds were stratified at 4° C for 3 days. Each batch of seeds was then inoculated in an Erlenmeyer flask (2 L volume) containing 300 mL growth medium (half-strength MS salts including vitamins, 1 % sucrose, 1 mM MES, brought to pH 5.9 with potassium hydroxide and autoclaved). For each experiment, six flasks were incubated at 25° C in an orbital shaker at 110 rpm for seven days with a photoperiod of 16 h light/8 h dark.

Two-phase partitioning (2PP)

Plantlets grown in liquid medium were drained with a strainer, rinsed with distilled water and gently blotted dry. All the subsequent procedures were carried out on ice. 80 g of fresh material was homogenized with 240 mL homogenization buffer (100 mM Hepes pH 7.5, 0.2 % N-Z-amino B, 250 mM sorbitol, 10 % Glycerol, 5 mM EDTA free acid, 5 mM Ascorbic acid, 0.6 % PVP K-25, 1 mM DTT) by a blender (PHD, Omni International, USA). Anti-protease and anti-phosphatase tablets (Complete protease inhibitor cocktail and PhosSTOP phosphatase inhibitor cocktail, Roche) were included in the buffer, according to the manufacturer's instructions. The homogenate was filtered through Miracloth (Millipore) and centrifuged at 10,000 rcf for 15 min at 4° C to remove cell debris. The supernatant was filtered through a 10 µm nylon mesh and

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3 centrifuged at 85,000 rcf for 1 h at 4° C to collect microsomes. Pellet was resuspended in 28 mL
4 resuspension buffer (5 mM potassium phosphate buffer pH 7.8, 0.33 M sucrose, 3 mM KCl, 0.1
5 mM EDTA) with the addition of antiprotease and antiphosphatase tablets. An aliquot of the total
6 microsomal preparation (Pre2PP) was retained. Two-phase partitioning⁹ as carried out by
7 loading 7 mL microsomes in tubes containing 21 g phases (6 % dextran, 6 % PEG 3350, 250
8 mM sucrose, 5 mM potassium phosphate buffer pH 7.8, 4 mM potassium chloride, 1 mM DTT,
9 50 µM EDTA). Two rounds of phase partitioning were performed. The PM-enriched upper
10 phases were then diluted with 150 mL resuspension buffer and centrifuged at 85,000 g for 1 h at
11 4° C. The pellet was dissolved in 3 mL re-suspension buffer and stored on ice. An aliquot of the
12 preparation (Post-2PP) was retained and the rest processed by FFE.
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Free-flow electrophoresis

The two-phase partitioning yielded 250 to 500 mg protein from 80 to 100 g of Arabidopsis seedlings (fresh weight). These enriched plant membranes samples were separated by free-flow electrophoresis based on previous approaches²² with slight modifications. The electrophoresis was performed using continuous zone electrophoresis-FFE (ZE-FFE) using a FFE System (BD Diagnostics). The setup employed the following conditions; 0.5 mm spacers for the separation chamber with 0.8 mm filter strips, the system was run in a horizontal position and cooled to 8° C. For Arabidopsis plasma membrane purifications, buffers were prepared as previously outlined²²,²³. Separation was achieved using 700 V, which resulted in a current of 105 to 115 mA. The media injection speed was 200 mL h⁻¹, and samples were injected at 1500 µL h⁻¹. Fractions were collected in pre-cooled 2 mL 96-well plates. Sample separation and system performance was assessed by monitoring protein distribution in the 96-well plates by measuring absorbance at 280

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3 nm with a SpectraMax Plus384 (Molecular Devices) microplate reader. Fractions corresponding
4 to the plasma membrane peak were recovered by centrifugation at 100,000 x g for 1h at 4° C
5 using a Beckman Coulter ultracentrifuge and a Type 70.1 Ti rotor. Pellets were re-suspended in
6 10 mM Tris-HCl (pH 7.5) and protein yield estimated using the Coomassie Plus (Bradford)
7 Protein Assay (Thermo Scientific). Aliquots were either immediately digested with trypsin for
8 analysis by mass spectrometry or were kept at -80° C until required.
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20 ***Immunoblotting***

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22 Protein samples (2 to 5 µg) from the purification process and FFE fractions were separated on 10
23 % NuPAGE® Bis-Tris Precast Gels (Life Technologies). Proteins were transferred to Hybond
24 ECL nitrocellulose membranes (GE Healthcare) using a Criterion™ Blotter (Bio-Rad) at 300
25 mA for 2 hours at 4° C. The antibodies employed were Anti-Calreticulin (CRT1, Abcam,
26 ab2907); Anti-plasma membrane H⁺-ATPase (AHA1, Agrisera, AS07 260); Anti-D1 protein of
27 PSII (PsbA), Agrisera, AS05 084); Anti-Voltage-dependent anion-selective channel protein 1
28 (VDAC1, Agrisera, AS07 212); Anti-cytosolic fructose-1,6-bisphosphatase (cFBPase, Agrisera,
29 AS04 043). All dilutions for primary antibodies were undertaken according to instructions by the
30 manufacturer. Detection was performed using a peroxidase-linked secondary antibody Anti-
31 Rabbit IgG (Sigma-Aldrich) diluted 1:20,000 and using SuperSignal West Dura Extended
32 Duration Substrate (Thermo Scientific). Images were captured using a BioSpectrum Imaging
33 System (UVP).
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53 ***Transient localization by particle bombardment***

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All clones for the co-localization experiments of FFE PM proteome candidate proteins were synthesized based on TAIR10 CDS models containing *attB* Gateway® sites (Forward: 5'-GGGGACAAGTTGTACA~~AAAAAA~~AGCAGGCTTCACC-3' and Reverse: 5'-GGGGACCACTTGTACAAGAAAGCTGGGTC-3') and without stop codons. Gateway® entry clones were created using synthesized genes and pDONR221-f1²⁴ after a BP reaction with Gateway® BP Clonase™ II Enzyme Mix (Life Technologies) following the manufacturer's protocol. Expression clones were created with the entry clones and pBullet destination vectors pBullet-pm-c and pBullet-pm-n²⁵ using the Gateway® LR Clonase™ II enzyme mix (Life Technologies) following manufacturer's protocol. Particle bombardment was performed based on previously described procedures²⁵. About 16 to 24 hours after bombardments, the onion epidermal cells were visualized under a Zeiss LSM 710 (Carl Zeiss) following previously outlined methods. Plasmolysis of cells was induced using 0.75 M mannitol treatment for 10 minutes. Image analysis and processing (scale bar, brightness, and contrast) were performed using the ImageJ software (Version 1.6r)²⁶.

Trypsin digestion for LC-MS/MS

Samples to be analyzed by mass spectrometry (equivalent to 5µg protein) were digested with trypsin (1:10 w/w) overnight at 37° C in 10 mM Tris-HCl (pH 7.5) and 50 % methanol²⁷. Resultant tryptic peptides were purified and concentrated using C₁₈ Ultra-Micro TipColumns (Harvard Apparatus).

Mass Spectrometry

Digested samples (approximately 1 ug) were analyzed by LC-MS/MS by nano-ESI-Q-TOF (TripleTOF® 5600 System, AB SCIEX) coupled to an Eksigent nano LC system (AB Sciex). Peptide samples were injected onto a Pepmap100 μ-guard column (Dionex-LC Packings, Sunnyvale, CA) via a Famos Autosampler (Dionex-LC Packings, Sunnyvale, CA) and washed for 10 minutes with Buffer A (2 % acetonitrile, 0.1 % formic acid) flowing at 15 μL/min. Peptides were eluted onto an Acclaim Pepmap100 C18 column (75 μm x 150 mm, 300 nL/min flow rate; Dionex-LC Packings) and into the TripleTOF 5600 via a gradient consisting of initial starting condition of 5 % buffer B (98 % acetonitrile, 0.1 % formic acid) increasing B to 35 % B over 60 minutes. Subsequently, B was increased to 90 % over three minutes and held for 15 minutes followed by a ramp back down to 5 % B over three minutes where it was held for 15 minutes to re-equilibrate the column to the original condition. Peptides were introduced to the mass spectrometer from the LC using a Nanospray III source (AB SCIEX) with a nano-tip emitter (New Objective, Woburn, MA) operating in positive-ion mode (2400 V). The data were acquired with Analyst TF 1.5.1 operating in information dependent acquisition (IDA) mode whereby after a 250 ms scan the twenty most intense ions (charge states 2 to 5) within 400 to 1600 m/z mass range above a threshold of 150 counts were selected for MS/MS analysis. MS/MS spectra were collected using TOF Resolution Mode: High Resolution with the quadrupole set to UNIT resolution and rolling collision energy to optimize fragmentation. MS/MS spectra were scanned from 100 to 1600 m/z and were collected for a total accumulation time of 50 ms. selected precursor ions were excluded for 16 seconds following MS/MS acquisition.

Data Analysis for Q-TOF Mass Spectrometry

The raw data were processed with the ProteinPilot Software package v.4.0 (AB Sciex, Foster City, CA) and matched with the Paragon Algorithm²⁸ against the Arabidopsis protein set (TAIR10) containing contaminants (keratin, trypsin, BSA) and comprised 35,397 total sequences comprising 14,487,047 residues. The Paragon Method employed standard settings with the instrument set as ‘TripleTOF 5600’ resulting in initial search parameters of 0.05 Da (MS) and 0.1 Da (MS/MS). The detected Protein Threshold was set at 99 % (Unused ProtScore [Conf] > 2.0) and a Thorough ID was applied for the Search Effort. The data processing and matching by ProteinPilot results in recalibration of data, which were subsequently exported as MGF Peaklist(s) for high confidence data matching. The recalibrated data were interrogated with the Mascot search engine version 2.3.02 (Matrix Science) with a peptide tolerance of \pm 50 ppm and MS/MS tolerance of \pm 0.1 Da; variable modification was Oxidation (M); up to one missed cleavage for trypsin; and the instrument type was set to ESI-QUAD-TOF. Searches were performed against Arabidopsis proteins (TAIR10 database containing contaminants), as detailed above. A false discovery rate and ions score or expected cut-off was calculated for each experiment using the Decoy feature of Mascot on the MS/MS Ions Search interface. A significance threshold corresponding to a false discovery rate of \leq 5 % ($p<0.05$) was used to determine the ‘Ions score or expect cut-off’ for peptide matches. As a result, ‘Ions score or expect cut-off values’ of 29 (AtPM-1a), 28 (AtPM-1b), 27 (AtPM-2a) and 28 (AtPM-2b) were used. Ions score is $-10\log(P)$, where P is the probability that the observed match is a random event. Ions scores \geq than those stated indicate identity or extensive homology ($p<0.05$). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium²⁹ via the PRIDE partner repository³⁰ with the dataset identifier PXD001795 and 10.6019/PXD001795.

Spectral counting and analysis

For each FFE fraction, all spectral counts (total high confidence peptide matches) assigned to each unique, non-redundant protein were summed, then expressed as a percentage of the total number of spectral counts in that fraction. For the FFE PM proteome, proteins were rejected if they had fewer than 5 SPCs summed across all fractions used to define AtPM-1 and AtPM-2, as low SPCs tend to be highly variable³¹. To consolidate SPCs from different FFE fractions, total %SPC was calculated by summing the SPCs for each protein across all PM FFE fractions used to define AtPM-1 and AtPM-2, then calculating %SPC using the summed SPC for all proteins in all fractions as the denominator. These percentages were then averaged across biological replicates, giving the total %SPC for the PM proteome. Proteins were annotated using the GO Plant Slim annotations from TAIR³², and SUBA consensus subcellular localization²¹. The data were also manually compared to various published data sets, including proteins detected in previously generated Arabidopsis PM proteomes^{13, 33-36}; predicted GPI anchored^{37, 38}, palmitoylated³⁹ or myristoylated proteins⁴⁰; proteins associated with the cell wall⁴¹, plasmodesmata (PD)⁴² or detergent resistant membranes (DRM)⁴³; proteins potentially, or known to be, involved in cellulose synthesis/deposition⁴⁴; and proteins belonging to certain large protein groups like the receptor-like kinases (RLKs)⁴⁵, ABC transporters⁴⁶, and ribosomal proteins⁴⁷. Additionally, the presence of protein transmembrane domains or an N-terminal signal sequence was predicted using the transmembrane hidden Markov model (TMHMM) prediction algorithm⁴⁸.

Gene Expression Normalization

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3 Protein abundances were normalized using gene expression level. The Nottingham Arabidopsis
4 Stock Centre (NASC)-Array database of microarray experiments was searched to find suitable
5 expression data⁴⁹. The microarray data selected was generated using plants from comparable
6 growth conditions and sampling times⁵⁰. The data from three biological replicates was retrieved,
7 and expression levels were averaged for genes expressed in two or more replicates. The
8 expression of each gene was converted to a %expression (%exp), using the summed expression
9 of all genes in the array as the denominator. Protein abundance was normalized by dividing
10 protein %SPC by its %exp. The change in ranked abundance (Δ Rank) of each protein after
11 normalization was calculated by subtracting the rank of the protein after normalization from the
12 rank before normalization. The proteins with the highest or lowest Δ Rank were assessed to
13 determine the effect of the gene expression normalization.
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PM Confidence Scoring

A PM-confidence score was calculated for each protein by averaging three scores assigned in
each applicable annotation category (SUBA consensus, GO cellular component, and gene
expression normalization). For SUBA consensus²¹, proteins scored a 10 if they were annotated
as PM localized only, a 5 if annotated as PM plus some other annotation, and a 1 if not annotated
as PM. Similarly for GO annotations³², proteins with only a PM annotation were given a 10,
those with a PM annotation and 1 or 2 other cellular component annotations were given an 8,
those with a PM annotation and 3 or more other cellular component annotation were given a 3,
and those without any PM annotations were given a 1. For the gene expression comparison,
proteins moving the furthest up in rank after gene expression normalization (high Δ Rank) were
given a value of 10, those moving the furthest down in rank were given a 1, and intermediate

values were given intermediate scores. In all cases, confidence scores were not affected when a protein was not annotated or detected by one of these methods.

Results

Isolation of Arabidopsis plasma membranes by free-flow electrophoresis

Purification of plasma membrane by 2PP has been extensively employed in plants to provide enriched plasma membrane fractions for downstream analyses¹⁰. However the success of the technique can be highly variable and result in contaminated membrane fractions. The utilization of FFE as an extra purification strategy for organelles and vesicles has been well established^{22, 23}. Consequently we sought to exploit the capacity of FFE to isolate high purity plasma membrane fractions from photosynthetic plant material. Liquid-culture grown Arabidopsis seedlings were harvested and plasma membranes were enriched by 2PP. These fractions were then subjected to further purification by FFE. The majority of plasma membrane vesicles seem to migrate as an 8 to 10 fraction peak between fractions 50 and 65 when using the FFE conditions outlined in this study (Figure 1). This is appreciably further toward the cathode than other, potentially contaminating, organelles^{22, 51}, meaning FFE can further purify PM vesicles isolated by 2PP. In order to confirm that this 10-fraction peak corresponded to plasma membrane vesicles, antibodies raised against the plasma membrane marker H⁺-ATPase (AHA1) were used to profile post-FFE fractions (Figure 1A). The ability of the FFE to separate contaminating membranes from the plasma membrane after 2PP was assessed using antibodies raised against plastid (PsbA), mitochondria (VDAC-1), vacuole (V-PPase), ER (BiP2, CRT) and cytosol (UGPase, cFBPase) marker proteins. Plasma membrane vesicles were clearly enriched by 2PP and, qualitatively, FFE produced minimal further enrichment as assessed by AHA1. Other organelle markers were reduced in the FFE fractions. Mitochondrial and vacuolar membranes (VDAC1 and V-PPase) were barely detectable in the PM peak after FFE. Plastid membranes

were detected beyond the anodic edge of the main PM peak (Figure 1A), indicating efficient removal of plastidic contaminants during electrophoresis. In contrast, ER (BIP2 and CRT) and cytosolic (UGPase and cFBPase) proteins that persisted through 2PP were not substantially removed from plasma membrane vesicles during FFE (Figure 1B).

Characterization of the FFE plasma membrane proteome by mass spectrometry

Plasma membrane vesicles from FFE fractions of two independent biological replicates comprising the apex of the plasma membrane peak, as defined by the AHA marker (Figure 1A) were collected and harvested by ultracentrifugation (Figure S-1). A total of two adjacent fractions (53 and 54) from the first replicate (AtPM-1) and 2 pools of three fractions (51, 52, 53 and 54, 55, 56) from the second replicate (AtPM-2) were digested overnight with trypsin and analyzed by LC-MS/MS. Resultant spectral data from LC-MS/MS analyses of fractions were exported and analyzed using the Mascot search engine against *Arabidopsis* proteins. A strict approach to data matching was employed where only peptides with ions scores above the $p < 0.05$ cut-off were accepted and a bold red peptide match was required for each protein match. Given these criteria, only a single peptide was necessary for a protein match in each replicate. For the experiment AtPM-1, a total of 1,269 proteins were identified in fraction 53 (AtPM-1a) and 898 proteins in fraction 54 (AtPM-1b). While for experiment AtPM-2, a total of 782 proteins were identified in pooled fractions 51 to 53 (AtPM-2a) and 1,151 proteins in pooled fractions 54 to 56 (AtPM-2b). Details of matched peptide data for the four samples analyzed by LC-MS/MS are outlined in Table S-1. Data were manually curated to remove redundant protein matches. This resulted in the identification of 1416 (experiment AtPM-1) and 1316 (experiment AtPM-2) non-redundant protein matches in each of the two analyses. The number of proteins identified in

both experiments was 1,029, which represents the protein set enriched by FFE (Figure S-1), referred to as the FFE PM proteome. This combined set of 1,029 was further manually curated to ensure consistency between redundant protein matches between the four replicates (Table S-2). The redundant protein matches encompass proteins matched with identical peptides outlined in Table S-1. These proteins represent valid protein matches, many of them encoded by alternatively spliced transcripts (Table S-3).

Defining the FFE plasma membrane proteome

To assist in the classification of proteins identified in the FFE PM proteome (1,029 proteins), a PM confidence score was developed based on gene ontologies, subcellular localizations, gene expression, and manual curation. Gene expression normalization was performed by dividing the protein abundance (spectral counts, SPC) by its normalized transcript level derived from data from 7-day-old wild type *Arabidopsis* seedlings⁵⁰. While individual transcript and protein levels may only be weakly correlated, high transcript levels are expected to roughly correlate with higher protein presence and detection. Protein abundance was ranked from most to least abundant, before and after normalization to the transcript level of the corresponding gene. Subtraction of the ‘after-normalization’ rank from the ‘before-normalization’ rank on the protein abundance list yielded a Δrank value. High Δrank values revealed proteins disproportionately enriched in the FFE PM proteome, compared to transcript abundance. After gene expression normalization, proteins whose Δrank was most increased tended to be low-abundance proteins (Table S4). The top 10 % Δrank proteins had an average of 6 spectral counts. These likely represent proteins that were enriched by the purification methods, i.e. proteins that are localized to the PM. In contrast, the highly abundant and highly expressed family of H⁺ATPases (AHAs),

which had an average of 1143 total SPC, were largely unaffected by gene expression normalization (Table S-4).

Proteins targeted to the secretory pathway, either by transmembrane domains or N-terminal signal sequences, are expected to be well represented in the FFE PM proteome. Indeed, after gene expression normalization, 34 % of proteins in the top 10 % Δ rank list contained a transmembrane domain, while only a single contaminating ribosomal protein was found in the top 10 % Δ rank list (Figure 2). Conversely, ribosomal proteins comprised 46 % of the bottom 10 % Δ rank, indicating that relative to their high expression level, they were depleted by the combination of 2PP and FFE (Figure 2). Since PM proteins only represent a subset of proteins encoded by a seedling transcriptome, changes in ranked abundance after normalization suggest an incongruity between transcript and protein abundance after PM purification. The approach both highlights PM proteins and de-emphasizes contaminants. In addition to Δ rank after gene expression normalization, SUBcellular Arabidopsis database (SUBA) consensus localization scores²¹, and the gene ontology (GO) cellular component annotation from TAIR³² were also included in calculation of the PM confidence score. SUBA and GO categories were assigned a value between 1 and 10. A minimum PM-confidence score of 1 was assigned to annotations unrelated to PM, while a maximum score of 10 was assigned to annotated PM-associated proteins (Table S-4). Values from 1 to 10 were also used to generate PM-confidence scores for gene expression Δ rank (i.e. the top 10 % of the Δ rank list were given a score of 10, the next 10 % were given a score of 9, and so on). These three PM confidence scores from the different methods were averaged to generate a confidence score for each protein. The FFE PM proteome contains 91 annotated ribosomal proteins⁴⁷, with an average confidence score of 1.8, while the

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3 72 identified receptor-like kinases⁴⁵ had an average PM-confidence score of 8.6 (Table S-4).
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5 Thus, receptor-like kinases, which are likely PM-localized, had a high average PM-confidence
6 score, while ribosomal proteins, which are likely PM proteome contaminants, had low average
7 PM-confidence scores. Therefore, the PM-confidence score effectively separated potential
8 contaminants from genuine PM proteins (Figure 2, Table S-4).
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18 ***Curation of the FFE PM proteome***
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20 Proteins in the post-FFE plasma membrane proteome were assigned to functional and subcellular
21 categories using data from MapMan⁵² and SUBA²¹ in combination with the calculated PM-
22 confidence scores (Table S-2). After manual curation, a total of 449 proteins (44 %) could be
23 confidently assigned as PM proteins (Figure 3A). Proteins derived from contaminating
24 organelles constituted 32 % (328 proteins) of the 1,029 proteins identified. A collection of 155
25 proteins were assigned as having functional associations to the PM and included proteins
26 assigned as vesicle trafficking, cell organization, chaperones, 14-3-3 proteins and extracellular
27 proteins. Collectively these proteins comprised 15 % of the FFE PM proteome. Lastly, a total of
28 97 proteins (9 %) were unable to be assigned a clear function or subcellular location but many
29 contained high PM-confidence scores. This group was assigned “unclear”, indicating an unclear
30 function or subcellular location within the cell. Given the PM-enriched nature of the preparation,
31 many likely represent authentic PM-associated proteins. The contribution of contaminants to a
32 proteome is more accurately assessed when some measure of relative abundance is taken into
33 account. Organelle distribution in the FFE PM proteome was thus also estimated using average
34 spectral counts (Figure 3B). Although this approach only resulted in a marginal increase in the
35 proportion of proteins assigned to the PM (46 %), it significantly reduced the contribution of
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contaminants to less than a fifth of the proteome (19 %). This difference confirms that proteins from contaminating organelles tend to be reduced in abundance in the post-FFE PM fractions. PM-associated proteins were enriched, from 15 to 31 %, when analyzed by relative abundance in the samples. Proteins contributing to this increase categorized to the cell organization category including actin, tubulin, and annexins. The contribution of proteins allocated as “unclear” was reduced to 4 %, suggesting these either originate from contaminating membranes or are low abundance PM proteins.

Validation of the FFE plasma membrane proteome

A total of 30 separate studies have sought to purify and characterize the PM proteome from *Arabidopsis*²¹, however this study represents the first PM proteome utilizing FFE as an orthogonal purification strategy⁵. Of the 1,029 proteins, a total of 97 were allocated as an unclear location or category (Table S-2). Interestingly, 39 of these proteins had not previously been identified in any subcellular proteome, and many had been assigned a high PM-confidence score. While only contributing about 4 % of the total proteins identified, this is a noteworthy observation considering that over 126 targeted proteomes have been characterized in *Arabidopsis*, which cumulatively have identified nearly 8,000 proteins²¹. Consequently, we selected a number of candidates from the proteome for localization using fluorescent proteins (FP). These candidates were relatively abundant in our preparations based on SPC, had a range of PM confidence scores and had either had not been previously localized to the plasma membrane or had contradictory subcellular assignments from other studies. The gene products were cloned into the pBullet-pm vectors²⁵, to enable either C- or N-terminal yellow fluorescent

protein (YFP) co-localization, and fusion proteins localized using particle bombardment and confocal microscopy (Figure 4, Table 1).

A total of 14 candidates were selected, however only 13 successfully expressed the fluorescent marker (Table 1). Those tagged with C-terminal YFP all showed some co-localization with the PM marker protein PIP2A (Figure 4a to 4m). Localization to the plasma membrane was assessed by light microscopy using 0.75 M mannitol⁵³, resulting in shrinkage of the protoplast and detachment of the plasma membrane from the cell wall. In addition, Hechtian strands, representing areas of adhesion between the plasma membrane (and associated cytosol) and the cell wall, were consistently visible with 10 of the constructs (Figure 4). For proteins tagged with N-terminal YFP, 4 out of 13 co-localized with the plasma membrane marker (Figure 4n to 4z). The other 9 N-terminal fusions did not show any signal, which could be attributed to the presence of a predicted N-terminal signal sequence or lipid modification (Table 1). An examination of the structure of these localized proteins reveals that candidates include predicted membrane proteins with single transmembrane domains such as uncharacterized receptor-like kinases (Figures 4b, 4e, 4m), transporters (Figure 4g), and multi-pass proteins of unknown function (Figures 4d, 4i, 4j). Finally, although some of these PM candidates contained no predicted transmembrane domain, they co-localized successfully with the PM marker (Figures 4a, 4c, 4f, 4h, 4k, 4l). Overall, the transient localization demonstrates that the combination of 2PP coupled to FFE identified many unknown bone fide PM-localized proteins.

Discussion

The plasma membrane represents a key selective barrier between the cell and its environment. Through the plasma membrane, plant cells sense their environment, deposit and modify their cell wall, and exchange nutrients and solutes. Thus, the plasma membrane proteome must be finely tuned to these situations. Furthermore, the plasma membrane proteome will be remodeled during development and in response to changing biotic and abiotic conditions. Coupling 2PP plasma membrane enrichment with FFE to remove contaminating membranes resulted in the identification of 1,029 proteins. Proteome confidence was analyzed by normalizing spectral counts to gene expression data, and using subcellular locations and functional annotations. This PM proteome contains over 700 PM-associated proteins with many abundant yet uncharacterized from one of the most intensively studied plant systems, *Arabidopsis* seedlings. Employing various data sources we developed a PM-confidence score and validated the PM localization for 13 candidates. These data represent a useful resource for researchers studying a variety of processes, including plant cell homeostasis, cell wall synthesis, cell signaling, vesicle trafficking, and biotic and abiotic responses.

Comparison of the FFE PM proteome with other *Arabidopsis* PM proteomes

The PM is a site of interest in terms of protein modification and protein response to the external environment. Consequently, a number of proteomic studies have previously been conducted on the plasma membrane. Many of these have been performed on a specific protein sub-population such as phosphorylated or GPI-anchored proteins^{11, 14, 16, 37}. Nevertheless, multiple general proteomic surveys of the PM exist, enabling meaningful comparisons with the current study and

thus an assessment of its contribution to uncovering new knowledge on the Arabidopsis PM (Table 2).

The PM has a very distinct migration profile during FFE which allows it to be separated from contaminating membranes. High protein identification numbers and high purity levels were therefore expected when a single round of 2PP was combined with FFE. The current study is comparable in protein numbers with other PM studies^{12, 13, 19} that have employed 2PP purification with data curation (Table 2). While no single previous 2PP proteome resulted in complete overlap with proteins identified in this study (Table 2), for the curated PM studies comprising 1875 non-redundant proteins^{12, 13, 19, 33, 35}, nearly 80 % were also identified in the our curated FFE PM proteome. The decision not to adopt detergent and/or carbonate washes after 2PP, as is commonly adopted with this approach^{12, 13}, could explain these differences. In fact a number of these proteins have not been identified by any subcellular proteomic study in Arabidopsis, yet have clear PM associated roles, this includes the Rho GTPase family member RIC4/ROP2 (AT1G20090.1) involved in the regulating polar expansion of cells, which has only been localized to the PM using fluorescent proteins⁵⁴. To examine how a non-2PP PM enrichment compared with our approach, we looked at the Arabidopsis PM proteome comprising 208 proteins defined by differential centrifugation incorporating localization of organelle proteins by isotope tagging (LOPIT)⁵⁵. A similar overlap was observed as was seen with the 2PP proteomes (Table 2), even though the LOPIT approach had incorporated a non-washed membrane sample in their workflow. However, given that the LOPIT approach seeks to determine multiple localizations from protein rich fractions, attaining sample depth may have limited their capacity to uncover a substantial PM proteome.

Finally, a comparison of proteins shared between AtPM-1 and AtPM-2 shows that the combination of 2PP and FFE does not appear to compromise experimental reproducibility. There are currently datasets from a further nine studies describing specific sub-populations of PM proteomes^{14, 16-18, 36, 38, 43, 56, 57}. Consequently, there is now a solid dataset of several hundred PM-resident proteins, so gains from further 2PP PM proteomes may be confirmatory or incremental. However, a current proteomics challenge is the identification of non-resident proteins with a functional role at the PM. As the PM has such a distinct electrophoretic migration profile⁵⁸ this suggests association of cytosolic proteins with the PM of both AtPM-1 and AtPM-2 vesicles is non-coincidental, indicating a functional association with the PM.

Gene expression normalization of proteomic data

Organellar proteomics is greatly limited by the quality of the fractionation and isolation procedure⁵⁹. While improved purification techniques can greatly reduce the number of contaminating proteins, some contaminant carry-over is inevitable, especially in an organelle with high protein turnover, such as the plasma membrane. In these cases, it is useful to identify proteins that are enriched or depleted by the purification procedure. Here, gene expression normalization was employed to enhance the signal to noise ratio in proteomic data. This method can reveal proteins that are enriched by organelle purification, relative to the protein levels predicted from the whole-cell transcriptome. Detecting a protein more often than its gene expression level would predict implies that the protein is enriched by the purification procedure. This can also highlight proteins that are abundant in the organellar proteome, in spite of a comparatively low gene expression. Though differences in rates of transcription and protein

turnover mean that protein abundance does not strongly correlate to gene expression levels⁶⁰, highly expressed genes are more likely to produce more protein than genes with low expression. The power of such an approach (when also coupled to other relevant data) is highlighted by the fact that four of the six proteins with an “unclear” localization according to SUBA were localized to the PM by tagged fluorescent protein had PM confidence scores greater than 6.0.

The FFE PM proteome of Arabidopsis

The largest source of contaminants by abundance in the FFE PM proteome were cytosolic proteins. However, many cytosolic proteins undergo transient association with the PM, e.g. components of vesicle trafficking. Other cytosolic proteins, including cytoskeleton proteins such as tubulins and actin subunits, may be indirectly associated with the PM via other proteins. For example, cortical microtubules are associated with the PM through a variety of microtubule-associated proteins⁶¹. CLIP-associated protein (CLASP) is a microtubule-associated protein that was detected in the FFE PM proteome (AT2G20190.1). CLASP is required for microtubule association with the cell cortex, presumably via mediating microtubule-plasma membrane interactions⁶². CLASP has no predicted transmembrane domains and has only been identified on a few occasions in PM proteomes of Arabidopsis^{11, 63}. The CELLULOSE SYNTHASE-INTERACTIVE PROTEIN 1 (CSI1) protein (AT2G22125.1), which was also detected, can interact with both microtubules and plasma membrane-localized cellulose synthase enzymes⁶⁴,⁶⁵, thereby forming an indirect bridge between the cortical cytoskeleton and the PM. Recently, both the NETWORKED 3C (NET3C, AT2G47920) and VESICLE ASSOCIATED PROTEIN (VAP27a, AT3G60600.1) proteins were found in association with ER-PM contact sites, and with the actin cytoskeleton⁶⁶; although NET3C was not identified in this study, the VAP27 protein

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3 and another ER-PM contact site protein, SYNAPTOTAGMIN1 (SYT1, At2g20990)⁶⁷ were
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5 reproducibly detected in all fractions. Other protein links between the cytoskeleton and/or other
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7 cellular organelles with the PM likely exist in this data set, and as such it presents multiple
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9 candidates for further investigations.
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15 The quality of the FFE PM proteome is also reflected by the depth of proteins detected. Several
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17 key plasma membrane localized complexes have been completely identified, including
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19 components that are peripheral membrane proteins that undergo transient and reversible
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21 association with the plasma membrane. For example, representatives of all eight members of the
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23 octameric exocyst complex⁶⁸, which is involved in polarized secretion, are detected in this FFE
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25 PM proteome; this includes AT1G47550.1 (SEC3A), AT1G76850.1 (SEC5A) AT1G71820.1
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27 (SEC6), AT3G10380.1 (SEC8), AT5G12370.1 (SEC10), AT4G02350.1 (SEC15B),
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29 AT5G49830.1 (EXO84B) and AT5G03540.1 (EXO70A1). Interestingly, EXO70 is a subfamily
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31 encoded in Arabidopsis by 23 members, in this FFE PM proteome only one member (EXO70A1)
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33 was identified⁶⁹. A total of seven out of eight recently-identified components of the TPLATE
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35 complex, required for clathrin-mediated endocytosis in plants⁷⁰ were all abundant components
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37 in our FFE PM proteome; including AT3G01780.1 (TPLATE), AT3G50590.1 (TWD40-1),
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39 AT5G24710.1 (TWD40-2), AT2G07360.1 (TASH3), AT5G57460.1 (TML), AT1G20760.1
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41 (AtEH1), AT1G21630.1 (AtEH2), AT2G25430.1 (AtECA4) and AT4G32285.1 (CAP1). Only
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43 the 17 kDa subunit, AT1G15370 (LOLITA) was not identified. Finally, over a third (21) of the
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45 57 Rab GTPases encoded by Arabidopsis⁷¹ were identified in the FFE PM proteome. The Rab
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47 GTPases are broadly associated with cellular trafficking and are involved in mediating the
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49 successive steps associated with endomembrane trafficking as well as enabling vesicle docking
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3 ⁷². The range of Rab GTPases outlined here highlights the variety of endocytic compartments
4 interacting with the PM, as recent data indicates that Rab GTPases are each likely associated
5 with distinct vesicle cargo ⁷³. Generally, these complexes are transiently formed at the PM
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7 through the recruitment of a number of cytosolic components to the PM periphery. This indicates
8 that the PM isolation techniques employed here are sufficiently gentle to leave biologically
9 important protein complexes undisrupted, including protein complexes that are rich in peripheral
10 membrane proteins, rather than only transmembrane proteins.
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22 Among the classes of proteins detected in these experiments, proteins required for water and
23 proton transport are highly represented in these data. This include the aquaporins (PIPs) required
24 for water balance in the cell and the H⁺-ATPases (AHAs), which are involved in maintaining the
25 membrane potential at the plasma membrane for proton-gradient mediated co-transport,
26
27 controlling the pH balance of the cell and the apoplasm. We identified 11 of the 13 PIP family
28 members ⁷⁴, including AT3G61430.1 (PIP1;1), AT2G45960.1 (PIP1;2), AT1G01620.1 (PIP1;3),
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30 AT4G00430.1 (PIP1;4), AT4G23400.1 (PIP1;5), AT3G53420.1 (PIP2;1), AT2G37170.1
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32 (PIP2;2), AT2G37180.1 (PIP2;3), AT5G60660.1 (PIP2;4), AT2G39010.1 (PIP2;6),
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34 AT4G35100.1 (PIP2;7). In contrast, we only identified 4 of the 11 members of the Arabidopsis
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36 AHA family ⁷⁵, but this included the highly expressed *AHA1* (At2g18960.1) and *AHA2*
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38 (At4g30190.1) isoforms, which were both highly abundant in this PM proteome (based on
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40 protein identification scores).
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53 We also identified a high number of proteins that are associated with cell wall biosynthetic and
54 structural processes. This included four members of the glucan synthase-like (GSL) protein
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family, which are involved in the biosynthesis of callose, a β -1,3-glucan polymer that accumulates at the cell plate during cell division, at plasmodesmata, and during wounding or pathogen attack. Of the twelve members found in Arabidopsis, the following GSL members were associated with the FFE PM proteome, namely AT2G31960.1 (GSL3), AT4G03550.1 (GSL5), AT2G36850.1 (GSL8) and AT3G07160.1 (GSL10). The family members GSL5, GSL8 and GSL10 are well represented in the proteome (based on protein identification scores), and all have demonstrated roles in pollen development^{76, 77} with GSL8 and GSL10 also functioning during normal plant growth⁷⁸. Further cell wall related proteins include a total of nine glycoside hydrolases (GHs), some of which may be involved in reorganizing the cell wall in the elongating seedling. This included six GH17 family members; AT1G64760.1, AT4G31140.1, AT5G58090.1, AT5G56590.1, AT1G11820.1 and AT5G42720.1. Interestingly, members of the CAZy GH17 family contain a carbohydrate-binding module at the C-terminal⁷⁹; while a number of these Arabidopsis proteins identified here also appear to contain glycosylphosphatidylinositol (GPI) anchors³⁷. This could suggest a role for these proteins in PM-associated interactions with the cell wall involving remodeling and maturation processes. Such a role has already been suggested for KORRIGAN (KOR1, AT5G49720.1), a PM localized GH9 family member exhibiting endo-1,4- β -D-glucanase activity with an involvement in cellulose biosynthesis⁸⁰. KOR1 and AT1G75680.1, both GH9 family members, along with AT5G04885.1 (GH3) were also identified in the FFE PM proteome.

Not surprisingly, there was also evidence for subunits of the cellulose synthase (CESA) complex, responsible for the biosynthesis of cellulose. This included AT5G05170.1 (CESA3) and AT4G32410.1 (CESA1), both of which are core components in the biosynthesis of the primary

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3 cell wall in *Arabidopsis*^{81, 82}, as would be expected for material sourced from young seedlings.
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6 However, based on spectral counts for these proteins, both constituted minor components of this
7 FFE PM proteome (Table S-4). Furthermore, a glaring omission is a CESA6-related member,
8 which would seemingly be required for a fully functional CESA complex⁸³. These observations
9 indicate that either the CESA complex has a strong link with the nascent cell wall, leading it to
10 partition with the wall fraction, or a strong interaction with internal structural components, such
11 as microtubules⁸⁴. Interestingly, we also identified several related GT2 clade members, namely
12 two cellulose synthase-like (Csl) proteins AT1G55850.1 (CslE1) and AT2G32540.1 (CslB4).
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15 The functional roles of these GT2 clade members is unclear⁸⁵, however proteomic surveys
16 indicate they are likely localized to the PM^{11, 20}, implicating these clade members in PM
17 associated cell wall biosynthetic processes, in contrast to other Csl clades which appear to be
18 Golgi localized⁸⁶⁻⁸⁸.
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21 Finally, a collection of cell wall-associated proteins were also identified, highlighting the
22 intimate relationship between the cell wall and the PM. This comprised six highly abundant
23 fasciclin-like arabinogalactan proteins (FLAs), including AT4G12730.1 (FLA1), AT1G03870.1
24 (FLA9), AT2G04780.1 (FLA7), AT5G44130.1 (FLA13), AT2G45470.1 (FLA8) and
25 AT5G55730.1 (FLA1). The fasciclin domain is an extracellular module which appears to play a
26 role in protein-protein interactions and cell adhesion in other biological systems⁸⁹. In plants,
27 some FLAs are required for cell wall deposition^{90, 91}. However, they have also been
28 hypothesized to play a role in PM-cell wall adhesion via their PM-localized GPI anchor and their
29 extracellular glycosylated domain. The *Arabidopsis* FLAs are comprised of 21 members and are
30 expressed throughout plant development⁹². Here we have identified a group of six members that
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3 are highly abundant in the FFE PM proteome, which provides candidates for FLAs that may be
4 critical for primary cell wall biosynthesis or for PM-cell wall adhesion in seedlings.
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10 The identification of multiple and highly expressed PIPs, AHAs, FLAs and other cell wall
11 associated proteins would be expected, since the source tissue for these experiments was young
12 seedlings, which are dominated by rapidly expanding cells. These growing cells require high
13 expression of cell wall synthesis and cell expansion machinery. Thus, many of the
14 uncharacterized proteins detected in these experiments may represent cell wall synthesis or
15 expansion components. Indeed, there is significant overlap between many of the proteins
16 detected in this plasma membrane proteome and the Arabidopsis homologs to proteins detected
17 in pull-down experiments with the cellulose synthase enzymes from poplar⁹³. Over one-third of
18 the putative interacting partners detected in crude microsomal fractions have homologs present in
19 the FFE PM proteome. The overlap between these two data sets represents exciting candidates
20 for further studies of plant cell wall synthesis and plant growth.
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Conclusion

The future of proteomic studies is the progression from the mere identification of protein inventories in a given organism, cell type or organelle to “functional” proteomics, i.e. assessing how protein identity and abundance varies in different conditions or following treatments. While a few studies have succeeded in demonstrating the effect of biotic and abiotic stresses on plasma membrane proteomes in plants⁹⁴, studies of changing proteomes in response to nutrients or identification of functional subdomains of plant plasma membranes have not yet been possible. By delivering high-purity PM isolates whilst incurring minimal loss of yield, the FFE technique has potential to advance functional analysis of the PM. In this study the capacity of FFE to enable high resolution purification of vesicles from a PM-enriched sample was demonstrated using liquid-grown *Arabidopsis* seedlings. This makes an excellent system for future proteomic studies because it is genetically accessible and seedlings can easily be subjected to a variety of controlled treatments.

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3 **Supporting Information Available**
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10 **Table S-1.** Mascot search results for AtPM-1a, AtPM-1b, AtPM-2a and AtPM-1a.
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12 **Table S-2.** Annotated FFE PM proteome.
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14 **Table S-3.** Redundant protein matches identified in the FFE PM proteome.
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16 **Table S-4.** Detailed data matrix used to generate PM confidence score.
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18 **Figure S-1.** Schematic workflow used to determine the FFE PM proteome.
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Conflict of Interest Disclosure

The authors declare no competing financial interest.

References

1. Kota, U.; Goshe, M. B., Advances in qualitative and quantitative plant membrane
2 proteomics. *Phytochemistry* **2011**, 72, 1040-1060.
3. Komatsu, S., Plasma membrane proteome in Arabidopsis and rice. *Proteomics* **2008**, 8,
4 4137-4145.
5. Tan, S.; Tan, H. T.; Chung, M. C., Membrane proteins and membrane proteomics.
6 *Proteomics* **2008**, 8, 3924-3932.
7. Chevalier, A. S.; Bienert, G. P.; Chaumont, F., A new LxxxA motif in the transmembrane
8 Helix3 of maize aquaporins belonging to the plasma membrane intrinsic protein PIP2 group is
9 required for their trafficking to the plasma membrane. *Plant Physiol.* **2014**, 166, 125-138.
10. Yadeta, K. A.; Elmore, J. M.; Coaker, G., Advancements in the analysis of the
11 Arabidopsis plasma membrane proteome. *Front. Plant Sci.* **2013**, 4, 86.
12. Vertommen, A.; Panis, B.; Swennen, R.; Carpentier, S. C., Challenges and solutions for
13 the identification of membrane proteins in non-model plants. *J. Proteomics* **2011**, 74, 1165-1181.
14. Hodges, T. K.; Mills, D., Isolation of the plasma membrane. *Method. Enzymol.* **1986**,
15 118, 41-54.
16. Bardy, N.; Carrasco, A.; Galaud, J. P.; Pont-Lezica, R.; Canut, H., Free-flow
17 electrophoresis for fractionation of *Arabidopsis thaliana* membranes. *Electrophoresis* **1998**, 19,
18 1145-1153.
19. Santoni, V., Plant plasma membrane protein extraction and solubilization for proteomic
20 analysis. In *Methods Mol. Biol.*, Thiellement, H.; Zivy, M.; Dmerval, C.; Machin, V., Eds.
21 Humana Press: New York, 2007; Vol. 355, pp 93-109.

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2
3 10. Larsson, C.; Widell, S.; Sommarin, M., Inside-out plant plasma-membrane vesicles of
4 high-purity obtained by aqueous 2-phase partitioning. *FEBS Lett.* **1988**, 229, 289-292.
5
6
7 11. Benschop, J. J.; Mohammed, S.; O'Flaherty, M.; Heck, A. J. R.; Slijper, M.; Menke, F. L.
8 H., Quantitative phosphoproteomics of early elicitor signaling in Arabidopsis. *Mol. Cell.*
9 *Proteomics* **2007**, 6, 1198-1214.
10
11
12 12. Elmore, J. M.; Liu, J.; Smith, B.; Phinney, B.; Coaker, G., Quantitative proteomics
13 reveals dynamic changes in the plasma membrane during Arabidopsis immune signaling. *Mol.*
14 *Cell. Proteomics* **2012**, 11, M111 014555.
15
16
17 13. Marmagne, A.; Ferro, M.; Meinnel, T.; Bruley, C.; Kuhn, L.; Garin, J.; Barbier-Brygoo,
18 H.; Ephritikhine, G., A high content in lipid-modified peripheral proteins and integral receptor
19 kinases features in the Arabidopsis plasma membrane proteome. *Mol. Cell. Proteomics* **2007**, 6,
20 1980-1996.
21
22
23 14. Nühse, T. S.; Stensballe, A.; Jensen, O. N.; Peck, S. C., Large-scale analysis of in vivo
24 phosphorylated membrane proteins by immobilized metal ion affinity chromatography and mass
25 spectrometry. *Mol. Cell. Proteomics* **2003**, 2, 1234-1243.
26
27
28 15. Schindler, J.; Nothwang, H. G., Aqueous polymer two-phase systems: Effective tools for
29 plasma membrane proteomics. *Proteomics* **2006**, 6, 5409-5417.
30
31
32 16. Nühse, T. S.; Stensballe, A.; Jensen, O. N.; Peck, S. C., Phosphoproteomics of the
33 Arabidopsis plasma membrane and a new phosphorylation site database. *Plant Cell* **2004**, 16,
34 2394-2405.
35
36
37 17. Elortza, F.; Nuhse, T. S.; Foster, L. J.; Stensballe, A.; Peck, S. C.; Jensen, O. N.,
38 Proteomic analysis of glycosylphosphatidylinositol-anchored membrane proteins. *Mol. Cell.*
39 *Proteomics* **2003**, 2, 1261-1270.
40
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42
43
44
45
46
47
48
49
50
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52
53
54
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3 18. Keinath, N. F.; Kierszniowska, S.; Lorek, J.; Bourdais, G.; Kessler, S. A.; Shimosato-
4 Asano, H.; Grossniklaus, U.; Schulze, W. X.; Robatzek, S.; Panstruga, R., PAMP (pathogen-
5 associated molecular pattern)-induced changes in plasma membrane compartmentalization reveal
6 novel components of plant immunity. *J. Biol. Chem.* **2010**, 285, 39140-39149.
7
8 19. Li, B.; Takahashi, D.; Kawamura, Y.; Uemura, M., Comparison of plasma membrane
9 proteomic changes of Arabidopsis suspension-cultured cells (T87 Line) after cold and ABA
10 treatment in association with freezing tolerance development. *Plant Cell Physiol.* **2012**, 53, 543-
11 554.
12
13 20. Mitra, S. K.; Walters, B. T.; Clouse, S. D.; Goshe, M. B., An efficient organic solvent
14 based extraction method for the proteomic analysis of Arabidopsis plasma membranes. *J.*
15 *Proteome Res.* **2009**, 8, 2752-2767.
16
17 21. Tanz, S. K.; Castleden, I.; Hooper, C. M.; Vacher, M.; Small, I.; Millar, H. a., SUBA3: a
18 database for integrating experimentation and prediction to define the SUBcellular location of
19 proteins in Arabidopsis. *Nucleic Acids Res.* **2013**, 41, 1185-1191.
20
21
22 22. Parsons, H. T.; Christiansen, K.; Knierim, B.; Carroll, A.; Ito, J.; Batth, T. S.; Smith-
23 Moritz , A. M.; Morrison, S.; McInerney, P.; Hadi, M. Z.; Auer, M.; Mukhopadhyay, A.;
24 Petzold, C. J.; Scheller, H. V.; Loqué, D.; Heazlewood, J. L., Isolation and proteomic
25 characterization of the Arabidopsis Golgi defines functional and novel targets involved in plant
26 cell wall biosynthesis. *Plant Physiol.* **2012**, 159, 12-26.
27
28 23. Eubel, H.; Heazlewood, J. L.; Millar, A. H., Isolation and subfractionation of plant
29 mitochondria for proteomic analysis. In *Plant Proteomics: Methods and Protocols*, Thiellement,
30 H.; Zivy, M.; Damerval, C.; Méchin, V., Eds. Humana Press Inc: Totowa, New Jersey, 2007;
31 Vol. 335, pp 49-62.
32
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49
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3 24. Lalonde, S.; Sero, A.; Pratelli, R.; Pilot, G.; Chen, J.; Sardi, M. I.; Parsa, S. A.; Kim, D.
4
5 Y.; Acharya, B. R.; Stein, E. V.; Hu, H. C.; Villiers, F.; Takeda, K.; Yang, Y.; Han, Y. S.;
6
7 Schwacke, R.; Chiang, W.; Kato, N.; Loque, D.; Assmann, S. M.; Kwak, J. M.; Schroeder, J. I.;
8
9 Rhee, S. Y.; Frommer, W. B., A membrane protein/signaling protein interaction network for
10
11 Arabidopsis version AMPv2. *Front Physiol* **2010**, 1, 24.
12
13
14 25. Lao, J.; Oikawa, A.; Bromley, J. R.; McInerney, P.; Suttangkakul, A.; Smith-Moritz, A.
15
16 M.; Plahar, H.; Chiu, T.-Y.; González Fernández-Niño, S. M.; Ebert, B.; Yang, F.; Christiansen,
17
18 K. M.; Hansen, S. F.; Stonebloom, S.; Adams, P. D.; Ronald, P. C.; Hillson, N. J.; Hadi, M. Z.;
19
20 Vega-Sánchez, M. E.; Loqué, D.; Scheller, H. V.; Heazlewood, J. L., The plant
21
22 glycosyltransferase clone collection for functional genomics. *Plant J.* **2014**, 79, 517-529.
23
24
25 26. Schneider, C. A.; Rasband, W. S.; Eliceiri, K. W., NIH Image to ImageJ: 25 years of
26
27 image analysis. *Nat. Methods* **2012**, 9, 671-675.
28
29
30 27. Russell, W. K.; Park, Z. Y.; Russell, D. H., Proteolysis in mixed organic-aqueous solvent
31
32 systems: applications for peptide mass mapping using mass spectrometry. *Anal. Chem.* **2001**, 73,
33
34 2682-2685.
35
36
37 28. Shilov, I. V.; Seymour, S. L.; Patel, A. A.; Loboda, A.; Tang, W. H.; Keating, S. P.;
38
39 Hunter, C. L.; Nuwaysir, L. M.; Schaeffer, D. A., The Paragon Algorithm, a next generation
40
41 search engine that uses sequence temperature values and feature probabilities to identify peptides
42
43 from tandem mass spectra. *Mol. Cell. Proteomics* **2007**, 6, 1638-1655.
44
45
46 29. Vizcaino, J. A.; Deutsch, E. W.; Wang, R.; Csordas, A.; Reisinger, F.; Rios, D.; Dianes,
47
48 J. A.; Sun, Z.; Farrah, T.; Bandeira, N.; Binz, P. A.; Xenarios, I.; Eisenacher, M.; Mayer, G.;
49
50 Gatto, L.; Campos, A.; Chalkley, R. J.; Kraus, H. J.; Albar, J. P.; Martinez-Bartolome, S.;
51
52 Apweiler, R.; Omenn, G. S.; Martens, L.; Jones, A. R.; Hermjakob, H., ProteomeXchange
53
54
55
56
57
58
59
60

provides globally coordinated proteomics data submission and dissemination. *Nat. Biotechnol.* **2014**, *32*, 223-226.

30. Vizcaino, J. A.; Cote, R. G.; Csordas, A.; Dianes, J. A.; Fabregat, A.; Foster, J. M.; Griss, J.; Alpi, E.; Birim, M.; Contell, J.; O'Kelly, G.; Schoenegger, A.; Ovelleiro, D.; Perez-Riverol, Y.; Reisinger, F.; Rios, D.; Wang, R.; Hermjakob, H., The Proteomics Identifications (PRIDE) database and associated tools: status in 2013. *Nucleic Acids Res.* **2013**, *41*, D1063-D1069.

31. Lundgren, D. H.; Hwang, S. I.; Wu, L.; Han, D. K., Role of spectral counting in quantitative proteomics. *Expert Rev. Proteomics* **2010**, *7*, 39-53.

32. Berardini, T. Z.; Mundodi, S.; Reiser, L.; Huala, E.; Garcia-hernandez, M.; Zhang, P.; Mueller, L. A.; Yoon, J.; Doyle, A.; Lander, G.; Moseyko, N.; Yoo, D.; Xu, I.; Zoeckler, B.; Montoya, M.; Miller, N.; Weems, D.; Rhee, S. Y.; Biology, P.; California, T. Z. B., Functional annotation of the Arabidopsis genome using controlled vocabularies. *Plant Physiol.* **2010**, *135*, 745-755.

33. Alexandersson, E.; Saalbach, G.; Larsson, C.; Kjellbom, P., Arabidopsis plasma membrane proteomics identifies components of transport , signal transduction and membrane trafficking. *Plant Cell Physiology* **2004**, *45*, 1543-1556.

34. Dunkley, T. P. J.; Hester, S.; Shadforth, I. P.; Runions, J.; Weimar, T.; Hanton, S. L.; Griffin, J. L.; Bessant, C.; Brandizzi, F.; Hawes, C.; Watson, R. B.; Dupree, P.; Lilley, K. S., Mapping the Arabidopsis organelle proteome. *Proc. Natl Acad. Sci. USA* **2006**, *103*, 6518-6523.

35. Marmagne, A.; Rouet, M.-A.; Ferro, M.; Rolland, N.; Alcon, C.; Joyard, J.; Garin, J.; Barbier-Bryggo, H.; Ephritikhine, G., Identification of new intrinsic proteins in Arabidopsis plasma membrane proteome. *Mol. Cell. Proteomics* **2004**, *3*, 675-691.

- 1
2
3 36. Nelson, C. J.; Hegeman, A. D.; Harms, A. C.; Sussman, M. R., A quantitative analysis of
4 Arabidopsis plasma membrane using trypsin-catalyzed (18)O labeling. *Mol. Cell. Proteomics*
5 **2006**, 5, 1382-1395.
6
7
8
9
10 37. Borner, G. H. H.; Lilley, K. S.; Stevens, T. J.; Dupree, P.; Centre, C., Identification of
11 glycosylphosphatidylinositol-anchored proteins in Arabidopsis. A proteomic and genomic
12 analysis. *Plant Physiol.* **2003**, 132, 568-577.
13
14
15
16
17 38. Elortza, F.; Mohammed, S.; Bunkenborg, J.; Foster, L. J.; Nu, T. S.; Brodbeck, U.; Peck,
18 S. C.; Jensen, O. N., Modification-specific proteomics of plasma membrane proteins:
19 Identification and characterization of glycosylphosphatidylinositol-anchored proteins released
20 upon phospholipase D treatment. *J. Proteome Res.* **2006**, 5, 935-943.
21
22
23
24
25
26 39. Hemsley, P. a.; Weimar, T.; Lilley, K. S.; Dupree, P.; Grierson, C. S., A proteomic
27 approach identifies many novel palmitoylated proteins in Arabidopsis. *New Phytol.* **2012**, 197,
28
29 805-814.
30
31
32
33 40. Podell, S.; Gribskov, M., Predicting N-terminal myristylation sites in plant proteins.
34 *BMC Genomics* **2004**, 5, 37.
35
36
37
38 41. Bayer, E. M.; Bottrill, A. R.; Walshaw, J.; Vigouroux, M.; Naldrett, M. J.; Thomas, C. L.;
39 Maule, A. J., Arabidopsis cell wall proteome defined using multidimensional protein
40
41 identification technology. *Proteomics* **2006**, 6, 301-311.
42
43
44 42. Fernandez-calvino, L.; Faulkner, C.; Walshaw, J.; Saalbach, G.; Benitez-alfonso, Y.;
45 Maule, A.; Bayer, E., Arabidopsis plasmodesmal proteome. *PLOS ONE* **2011**, 6, e18880.
46
47
48
49
50 43. Borner, G. H. H.; Sherrier, D. J.; Weimar, T.; Michaelson, L. V.; Hawkins, N. D.;
51 Macaskill, A.; Napier, J. A.; Beale, M. H.; Lilley, K. S.; Dupree, P., Analysis of detergent-
52
53
54
55
56
57
58
59
60

1
2
3 resistant membranes in Arabidopsis. Evidence for plasma membrane lipid rafts. *Plant Physiol.*
4
5 **2005**, 137, 104-116.
6
7

8 44. Mizrahi, E.; Mansfield, S. D.; Myburg, A. A., Cellulose factories: advancing bioenergy
9 production from forest trees. *New Phytol.* **2012**, 194, 54-62.
10
11

12 45. Shiu, S.-h.; Bleecker, A. B., Expansion of the receptor-like kinase / pelle gene family and
13 receptor-like proteins in Arabidopsis. *Plant Physiol.* **2003**, 132, 530-543.
14
15

16 46. Kang, J.; Park, J.; Choi, H.; Burla, B.; Kretzschmar, T.; Lee, Y.; Martinoia, E., Plant
17 ABC Transporters. *The Arabidopsis Book* **2011**, 9, 1-25.
18
19

20 47. Carroll, A.; Heazlewood, J. L.; Ito, J.; Millar, A. H., Systematic analysis of the
21 Arabidopsis cytosolic ribosome proteome by tandem mass spectrometry provides detailed
22 insights into its protein complement and their post-translational modification. *Mol. Cell.*
23
24 *Proteomics* **2008**, 7, 342-369.
25
26

27 48. Sonnhammer, E. L. L.; von Heijne, G.; Krogh, A., A hidden Markov model for predicting
28 transmembrane helices in protein sequences. *Proceedings of ISMB-98* **1998**, 6, 175-182.
29
30

31 49. Craigon, D. J.; James, N.; Okyere, J.; Higgins, J.; Jotham, J.; May, S., NASCArrays: a
32 repository for microarray data generated by NASC's transcriptomics service. *Nucleic Acids Res.*
33
34 **2004**, 32, D575-577.
35
36

37 50. Delker, C.; Pöschl, Y.; Raschke, A.; Ullrich, K.; Ettingshausen, S.; Hauptmann, V.;
38 Grosse, I.; Quint, M., Natural variation of transcriptional auxin response networks in *Arabidopsis*
39 *thaliana*. *Plant Cell* **2010**, 22, 2184-2200.
40
41

42 51. Canut, H.; Bauer, J.; Weber, G., Separation of plant membranes by electromigration
43 techniques. *J. Chromatogr. B* **1999**, 722, 121-139.
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

52. Thimm, O.; Blasing, O.; Gibon, Y.; Nagel, A.; Meyer, S.; Kruger, P.; Selbig, J.; Muller, L. A.; Rhee, S. Y.; Stitt, M., MAPMAN: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. *Plant J.* **2004**, 37, 914-939.

53. Campos-Soriano, L.; Gomez-Ariza, J.; Bonfante, P.; Segundo, B. S., A rice calcium-dependent protein kinase is expressed in cortical root cells during the presymbiotic phase of the arbuscular mycorrhizal symbiosis. *BMC Plant Biol.* **2011**, 11.

54. Fu, Y.; Li, H.; Yang, Z. B., The ROP2 GTPase controls the formation of cortical fine F-actin and the early phase of directional cell expansion during *Arabidopsis* organogenesis. *Plant Cell* **2002**, 14, 777-794.

55. Nikolovski, N.; Rubtsov, D.; Segura, M. P.; Miles, G. P.; Stevens, T. J.; Dunkley, T. P.; Munro, S.; Lilley, K. S.; Dupree, P., Putative glycosyltransferases and other plant Golgi apparatus proteins are revealed by LOPIT proteomics. *Plant Physiol.* **2012**, 160, 1037-1051.

56. Monneuse, J. M.; Sugano, M.; Becue, T.; Santoni, V.; Hem, S.; Rossignol, M., Towards the profiling of the *Arabidopsis thaliana* plasma membrane transportome by targeted proteomics. *Proteomics* **2011**, 11, 1789-1797.

57. Santoni, V.; Vinh, J.; Pflieger, D.; Sommerer, N.; Maurel, C., A proteomic study reveals novel insights into the diversity of aquaporin forms expressed in the plasma membrane of plant roots. *Biochem. J.* **2003**, 373, 289-296.

58. Canut, H.; Brightman, A.; Boudet, A. M.; Morre, D. J., Plasma-membrane vesicles of opposite sidedness from soybean hypocotyls by preparative Free-Flow Electrophoresis. *Plant Physiol.* **1988**, 86, 631-637.

59. Millar, A. H.; Taylor, N. L., Subcellular proteomics—where cell biology meets protein chemistry. *Front. Plant Sci.* **2014**, 5, 55.

- 1
2
3 60. Greenbaum, D.; Colangelo, C.; Williams, K.; Gerstein, M., Comparing protein
4 abundance and mRNA expression levels on a genomic scale. *Genome Biol.* **2003**, 4, 1-8.
5
6 61. Wasteneys, G. O.; Ambrose, J. C., Spatial organization of plant cortical microtubules:
7 close encounters of the 2D kind. *Trends Cell Biol.* **2009**, 19, 62-71.
8
9 62. Ambrose, J. C.; Wasteneys, G. O., CLASP modulates microtubule-cortex interaction
10 during self-organization of acentrosomal microtubules. *Mol. Biol. Cell* **2008**, 19, 4730-4737.
11
12 63. Zhang, Z. J.; Peck, S. C., Simplified enrichment of plasma membrane proteins for
13 proteomic analyses in *Arabidopsis thaliana*. *Proteomics* **2011**, 11, 1780-1788.
14
15 64. Gu, Y.; Kaplinsky, N.; Bringmann, M.; Cobb, A.; Carroll, A.; Sampathkumar, A.;
16 Baskin, T. I.; Persson, S.; Somerville, C. R., Identification of a cellulose synthase-associated
17 protein required for cellulose biosynthesis. *Proc. Natl Acad. Sci. USA* **2010**, 107, 12866-12871.
18
19 65. Mei, Y.; Gao, H. B.; Yuan, M.; Xue, H. W., The Arabidopsis ARCP protein, CSI1, which
20 is required for microtubule stability, is necessary for root and anther development. *Plant Cell*
21
22 **2012**, 24, 1066-1080.
23
24 66. Wang, P. W.; Hawkins, T. J.; Richardson, C.; Cummins, I.; Deeks, M. J.; Sparkes, I.;
25 Hawes, C.; Hussey, P. J., The Plant Cytoskeleton, NET3C, and VAP27 Mediate the Link
26 between the Plasma Membrane and Endoplasmic Reticulum. *Curr. Biol.* **2014**, 24, 1397-1405.
27
28 67. Pérez-Sancho, J.; Vanneste, S.; Lee, E.; McFarlane, H. E.; del Valle, A. E.; Valpuesta,
29 V.; Friml, J.; Botella, M. A.; Rosado, A., The Arabidopsis Synaptotagmin1 is enriched in
30 endoplasmic reticulum-plasma membrane contact sites and confers cellular resistance to
31 mechanical stresses. *Plant Physiol.* **2015**, 168, 132-U837.
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

- 1
2
3 68. Hala, M.; Cole, R.; Synek, L.; Drdova, E.; Pecenkova, T.; Nordheim, A.; Lamkemeyer,
4 T.; Madlung, J.; Hochholdinger, F.; Fowler, J. E.; Zarsky, V., An exocyst complex functions in
5 plant cell growth in *Arabidopsis* and tobacco. *Plant Cell* **2008**, 20, 1330-1345.
6
7
8
9
10 69. Zhang, Y.; Liu, C. M.; Emons, A. M.; Ketelaar, T., The plant exocyst. *J. Integr. Plant
11 Biol.* **2010**, 52, 138-146.
12
13
14
15 70. Gadeyne, A.; Sanchez-Rodriguez, C.; Vanneste, S.; Di Rubbo, S.; Zauber, H.; Vanneste,
16 K.; Van Leene, J.; De Winne, N.; Eeckhout, D.; Persiau, G.; Van De Slijke, E.; Cannoot, B.;
17 Vercruyse, L.; Mayers, J. R.; Adamowski, M.; Kania, U.; Ehrlich, M.; Schweighofer, A.;
18 Ketelaar, T.; Maere, S.; Bednarek, S. Y.; Friml, J.; Gevaert, K.; Witters, E.; Russinova, E.;
19 Persson, S.; De Jaeger, G.; Van Damme, D., The TPLATE adaptor complex drives clathrin-
20 mediated endocytosis in plants. *Cell* **2014**, 156, 691-704.
21
22
23
24
25
26
27
28
29 71. Rutherford, S.; Moore, I., The *Arabidopsis* Rab GTPase family: another enigma
30 variation. *Curr. Opin. Plant Biol.* **2002**, 5, 518-528.
31
32
33
34 72. Nielsen, E.; Cheung, A. Y.; Ueda, T., The regulatory RAB and ARF GTPases for
35 vesicular trafficking. *Plant Physiol.* **2008**, 147, 1516-1526.
36
37
38
39 73. Heard, W.; Sklenar, J.; Tome, D. F. A.; Robatzek, S.; Jones, A. M. E., Identification of
40 regulatory and cargo proteins of endosomal and secretory pathways in *Arabidopsis thaliana* by
41 proteomic dissection. *Mol. Cell. Proteomics* **2015**, 14, 1796-1813.
42
43
44
45
46 74. Jang, J. Y.; Kim, D. G.; Kim, Y. O.; Kim, J. S.; Kang, H. S., An expression analysis of a
47 gene family encoding plasma membrane aquaporins in response to abiotic stresses in
48 *Arabidopsis thaliana*. *Plant Mol. Biol.* **2004**, 54, 713-725.
49
50
51
52
53 75. Palmgren, M. G., Plant plasma membrane H⁺-ATPases: Powerhouses for nutrient uptake.
54
55 *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **2001**, 52, 817-845.
56
57
58
59
60

- 1
2
3 76. Enns, L. C.; Kanaoka, M. M.; Torii, K. U.; Comai, L.; Okada, K.; Cleland, R. E., Two
4 callose synthases, GSL1 and GSL5, play an essential and redundant role in plant and pollen
5 development and in fertility. *Plant Mol. Biol.* **2005**, 58, 333-349.
6
7 77. Chen, X. Y.; Liu, L.; Lee, E.; Han, X.; Rim, Y.; Chu, H.; Kim, S. W.; Sack, F.; Kim, J.
8 Y., The Arabidopsis callose synthase gene *GSL8* Is required for cytokinesis and cell patterning.
9
10
11
12
13
14
15
16
17 78. Töller, A.; Brownfield, L.; Neu, C.; Twell, D.; Schulze-Lefert, P., Dual function of
18 Arabidopsis glucan synthase-like genes *GSL8* and *GSL10* in male gametophyte development and
19 plant growth. *Plant J.* **2008**, 54, 911-923.
20
21
22
23
24
25 79. Barral, P.; Suarez, C.; Batanero, E.; Alfonso, C.; Alche, J. D.; Rodriguez-Garcia, M. I.;
26 Villalba, M.; Rivas, G.; Rodriguez, R., An olive pollen protein with allergenic activity, Ole e 10,
27 defines a novel family of carbohydrate-binding modules and is potentially implicated in pollen
28 germination. *Biochem. J.* **2005**, 390, 77-84.
29
30
31
32
33
34 80. Mansoori, N.; Timmers, J.; Desprez, T.; Kamei, C. L.; Dees, D. C.; Vincken, J. P.;
35 Visser, R. G.; Hofte, H.; Vernhettes, S.; Trindade, L. M., KORRIGAN1 interacts specifically
36 with integral components of the cellulose synthase machinery. *PLOS ONE* **2014**, 9, e112387.
37
38
39
40
41 81. Desprez, T.; Juraniec, M.; Crowell, E. F.; Jouy, H.; Pochylova, Z.; Parcy, F.; Hofte, H.;
42 Gonneau, M.; Vernhettes, S., Organization of cellulose synthase complexes involved in primary
43 cell wall synthesis in *Arabidopsis thaliana*. *Proc. Natl Acad. Sci. USA* **2007**, 104, 15572-15577.
44
45
46
47
48 82. Persson, S.; Paredez, A.; Carroll, A.; Palsdottir, H.; Doblin, M.; Poindexter, P.; Khitrov,
49 N.; Auer, M.; Somerville, C. R., Genetic evidence for three unique components in primary cell-
50 wall cellulose synthase complexes in Arabidopsis. *Proc. Natl Acad. Sci. USA* **2007**, 104, 15566-
51 15571.
52
53
54
55
56
57
58
59
60

- 1
2
3 83. Taylor, N. G., Cellulose biosynthesis and deposition in higher plants. *New Phytol.* **2008**,
4 178, 239-252.
5
6
7 84. Worden, N.; Wilkop, T. E.; Esteve, V. E.; Jeannotte, R.; Lathe, R.; Vernhettes, S.;
8 Weimer, B.; Hicks, G.; Alonso, J.; Labavitch, J.; Persson, S.; Ehrhardt, D.; Drakakaki, G., CESA
9 TRAFFICKING INHIBITOR inhibits cellulose deposition and interferes with the trafficking of
10 cellulose synthase complexes and their associated proteins KORRIGAN1 and
11 POM2/CELLULOSE SYNTHASE INTERACTIVE PROTEIN1. *Plant Physiol.* **2015**, 167, 381-
12 U609.
13
14
15 85. Yin, Y. B.; Huang, J. L.; Xu, Y., The cellulose synthase superfamily in fully sequenced
16 plants and algae. *BMC Plant Biol.* **2009**, 9.
17
18 86. Liepmann, A. H.; Wilkerson, C. G.; Keegstra, K., Expression of cellulose synthase-like
19 (Csl) genes in insect cells reveals that CslA family members encode mannan synthases. *Proc.*
20 *Natl Acad. Sci. USA* **2005**, 102, 2221-2226.
21
22 87. Dhugga, K. S.; Barreiro, R.; Whitten, B.; Stecca, K.; Hazebroek, J.; Randhawa, G. S.;
23 Dolan, M.; Kinney, A. J.; Tomes, D.; Nichols, S.; Anderson, P., Guar seed beta-mannan synthase
24 is a member of the cellulose synthase super gene family. *Science* **2004**, 303, 363-366.
25
26 88. Yin, L.; Verhertbruggen, Y.; Oikawa, A.; Manisseri, C.; Knierim, B.; Prak, L.; Jensen, J.
27 K.; Knox, J. P.; Auer, M.; Willats, W. G. T.; Scheller, H. V., The cooperative activities of
28 CSLD2, CSLD3, and CSLD5 are required for normal Arabidopsis development. *Mol. Plant*
29 **2011**, 4, 1024-1037.
30
31 89. Elkins, T.; Hortsch, M.; Bieber, A. J.; Snow, P. M.; Goodman, C. S., *Drosophila*
32 fasciclin-I is a novel homophilic adhesion molecule that along with fasciclin-III can mediate cell
33 sorting. *J. Cell Biol.* **1990**, 110, 1825-1832.
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

- 1
2
3 90. Harpaz-Saad, S.; McFarlane, H. E.; Xu, S. L.; Divi, U. K.; Forward, B.; Western, T. L.;
4 Kieber, J. J., Cellulose synthesis via the FEI2 RLK/SOS5 pathway and CELLULOSE
5 SYNTHASE 5 is required for the structure of seed coat mucilage in Arabidopsis. *Plant J.* **2011**,
6 68, 941-953.
7
8 91. MacMillan, C. P.; Mansfield, S. D.; Stachurski, Z. H.; Evans, R.; Southerton, S. G.,
9 Fasciclin-like arabinogalactan proteins: specialization for stem biomechanics and cell wall
10 architecture in Arabidopsis and Eucalyptus. *Plant J.* **2010**, 62, 689-703.
11
12 92. Johnson, K. L.; Jones, B. J.; Bacic, A.; Schultz, C. J., The fasciclin-like arabinogalactan
13 proteins of Arabidopsis. A multigene family of putative cell adhesion molecules. *Plant Physiol.*
14
15 2003, 133, 1911-1925.
16
17 93. Song, D.; Shen, J.; Li, L., Characterization of cellulose synthase complexes in Populus
18 xylem differentiation. *New Phytol.* **2010**, 187, 777-790.
19
20 94. Stanislas, T.; Bouyssie, D.; Rossignol, M.; Vesa, S.; Fromentin, J.; Morel, J.; Pichereaux,
21 C.; Monserrat, B.; Simon-Plas, F., Quantitative proteomics reveals a dynamic association of
22 proteins to detergent-resistant membranes upon elicitor signaling in tobacco. *Mol. Cell.*
23
24 *Proteomics* **2009**, 8, 2186-2198.
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
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3 **Table 1. Localization of selected proteins identified in the FFE PM proteome using particle bombardment.**

4 Subcellular interpretations were made from co-localization experiments of FFE PM proteome candidate proteins using pBullet-pm-c
5 and pBullet-pm-n vectors containing the plasma membrane marker (PIP2A; At3g53420). Prior localization information was obtained
6 from SUBA for subcellular proteomic (MS) and tagged fluorescent protein (FP).
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Key (Figure 4)	AGI	Location (C-YFP)	Location (N-YFP)	Assignment (Table S-2)	PM-confidence score	SUBA (MS)	SUBA (FP)	Final Location
a	AT1G12080.2	PM	no signal	unclear	6.00			PM
b	AT1G51940.1	PM	PM	unclear	9.67			PM
c	AT1G52100.1	weak PM	PM	unclear	4.00			unclear
d	AT1G73650.3	PM	PM	PM	7.33	Golgi		PM
e	AT1G80640.1	PM	no signal	unclear	9.33			PM
f	AT2G18360.1	PM	no signal	unclear	7.50			PM
g	AT2G38940.1	Put. PM	no signal	PM	7.67	PM		PM
h	AT3G16420.1	PM	no signal	unclear	1.00	multiple		PM
i	AT3G27390.1	Put. PM	no signal	PM	9.00	multiple		PM
	AT3G28040.1	no signal	no signal	unclear	8.67			n.d.
j	AT3G45600.1	PM	no signal	PM	9.33	PM		PM
k	AT4G11860.1	PM	no signal	PM	6.33	multiple		PM
l	AT4G21450.1	PM	no signal	PM	8.67	PM		PM
m	AT5G06320.1	PM	PM	PM	7.33	PM	unclear	PM

Table 2. The 2PP PM proteomes of Arabidopsis.

Enrichment Method	Total Protein Number	PM Proteins	PM Proteins Shared	Main contaminant groups	Reference
2PP + FFE	1029	701		cytosol, ER	This study
2PP	2302	1342	506	plastid, cytosol	¹²
2PP	658	580	194	cytosol, plastid	¹⁹
2PP	2021	n.d.	321*	plastid, cytosol	²⁰
2PP	446	446	226	cytosol	¹³
2PP	102	102	48		³⁵
2PP	304	222	123	cytosol, plastid, mitochondria	³³
LOPIT	1385	208	132	mixed organelle analysis	⁵⁵

*Total proteins used for calculation.

Figure Legends**Figure 1. An Arabidopsis seedling 2PP sample separated by FFE**

(A) Migration of 2PP derived Arabidopsis proteins during FFE measured by absorbance (280 nm), indicating the absorbance peak enriched in PM proteins at fractions 53 to 54 corresponding to sample AtPM-1. For FFE fractions 37-63, immunoblot analysis (2.5 µg protein) shows the abundance of PM (AHA) and plastid (PsbA) markers. The peak located at fraction 15 corresponds to the FFE gutter and likely comprises soluble protein electrophoresed from the membrane vesicles.

(B) Analysis of samples by immunoblotting (2.5 µg protein) from pre-enrichment (Pre-2PP), post enrichment by 2PP (Post-2PP) and after fractionation by FFE (Frac). Antibodies correspond to AHA (PM); PsbA (plastid); VDAC1 (mitochondria); V-PPase (vacuole); UGPase, cFBPase (cytosol) BiP2 and CRT (ER - endoplasmic reticulum).

Figure 2. Gene expression normalization (Δ rank) and the PM-confidence score.

The score can be used to discriminate between true PM proteins and contaminants. The percent of proteins containing a TMD/signal sequence, or that are part of the ribosomal protein complex, was determined for the highest scoring (top 10 %) and lowest scoring (bottom 10 %) proteins.

The total number of proteins is shown on top of the bar.

Figure 3. Subcellular distribution of the FFE PM proteome.

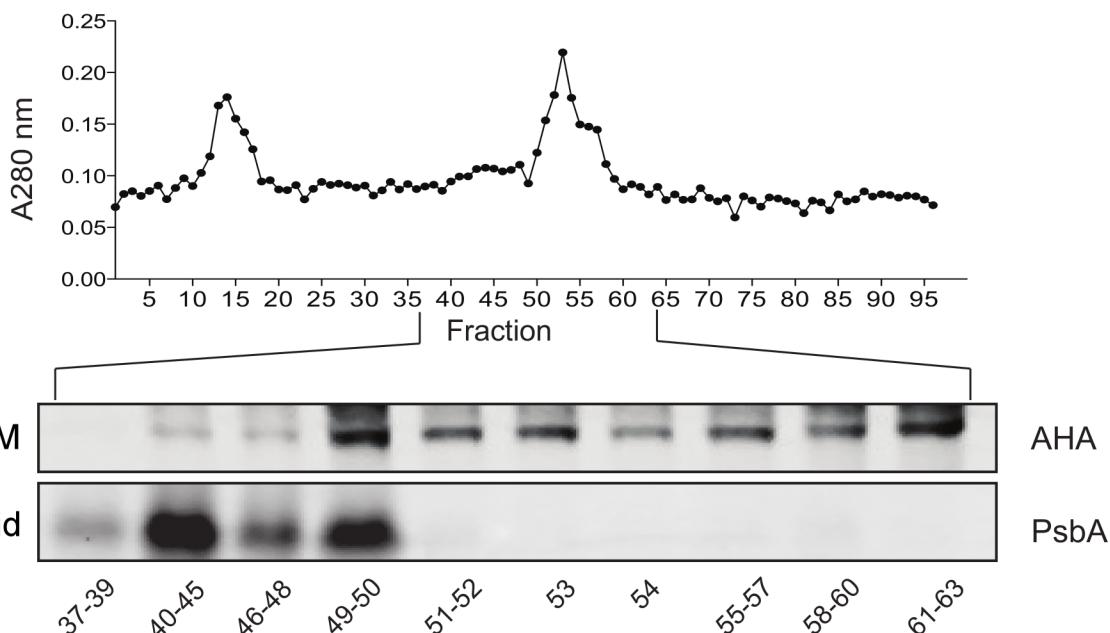
Distribution of the PM proteome according to the subcellular and functional annotations in Table S-2 (A) employing total protein numbers and (B) employing SPC. The sidebars show the proportion of functional classes that comprise the Contaminants (purple) or PM-Associated (green).

Figure 4. Localization of selected candidates from the FFE PM proteome by particle bombardment using N- and C-terminal yellow fluorescent protein (YFP) tags.

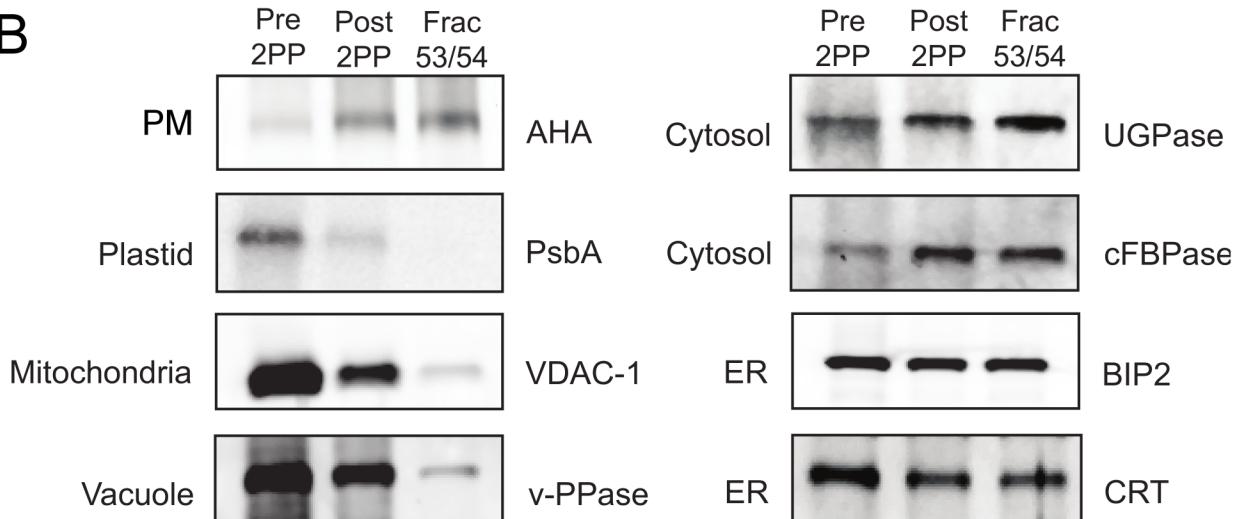
Subcellular localization of candidate proteins was performed using particle bombardment with the pBullet-pm-c and pBullet-pm-n vectors and examined after 16 to 24 hours. The columns labeled: 'pBullet-pm' indicates the PM marker PiP2a-ECFP (At3g53420), 'cYFP' are candidate

proteins with a C-terminal EYFP, ‘nYFP’ are candidate proteins with a N-terminal EYFP, ‘light’ is the transmitted light image, ‘merged’ indicates images overlays and ‘Hechtian’ indicates that Hechtian strands were detectable. Where Hechtian strands were not detected, a protein was assigned as putative PM (c, g, i). The cells showing signals using pBullet-pm-c were treated with 0.75 M mannitol for 10 minutes to induce plasmolysis (a - m). Of the 14 candidates fused to a C-terminal EYFP, 13 displayed PM localizations. A total of four candidates localized to the PM when fused to an N-terminal EYFP (o, p, q, z). The other 12 resulted in little or no signal using this construct. Localization results are summarized in Table 1. Scale bar = 20 μm for all images except ‘Hechtian’ where scale bar = 10 μm .

A



B



ACS Paragon Plus Environment

Figure 1

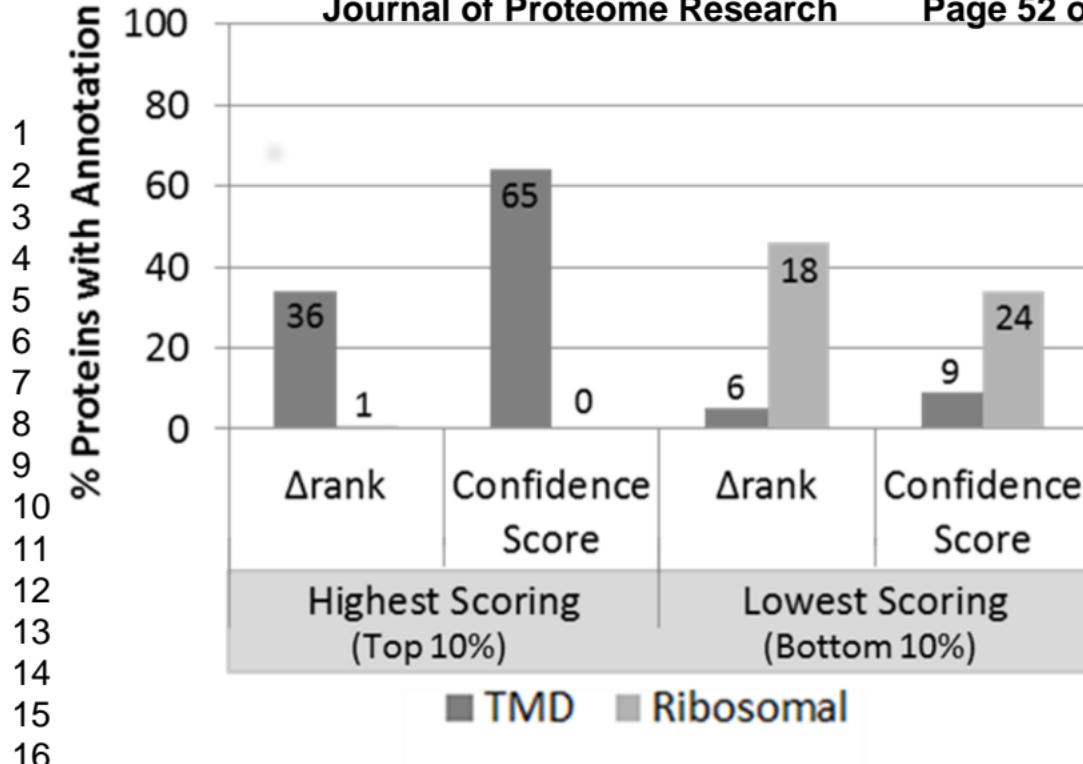


Figure 2

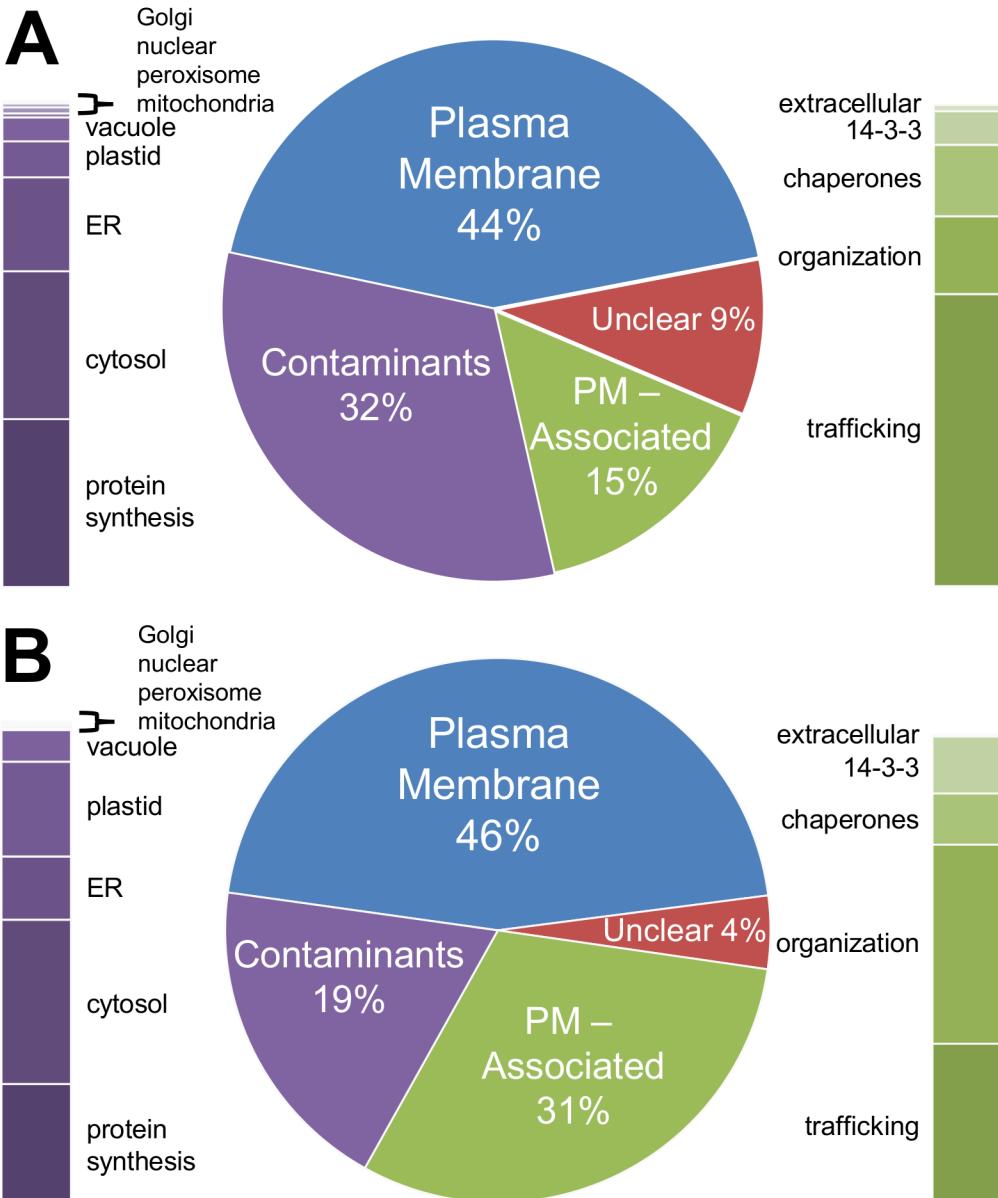


Figure 3

ACS Paragon Plus Environment

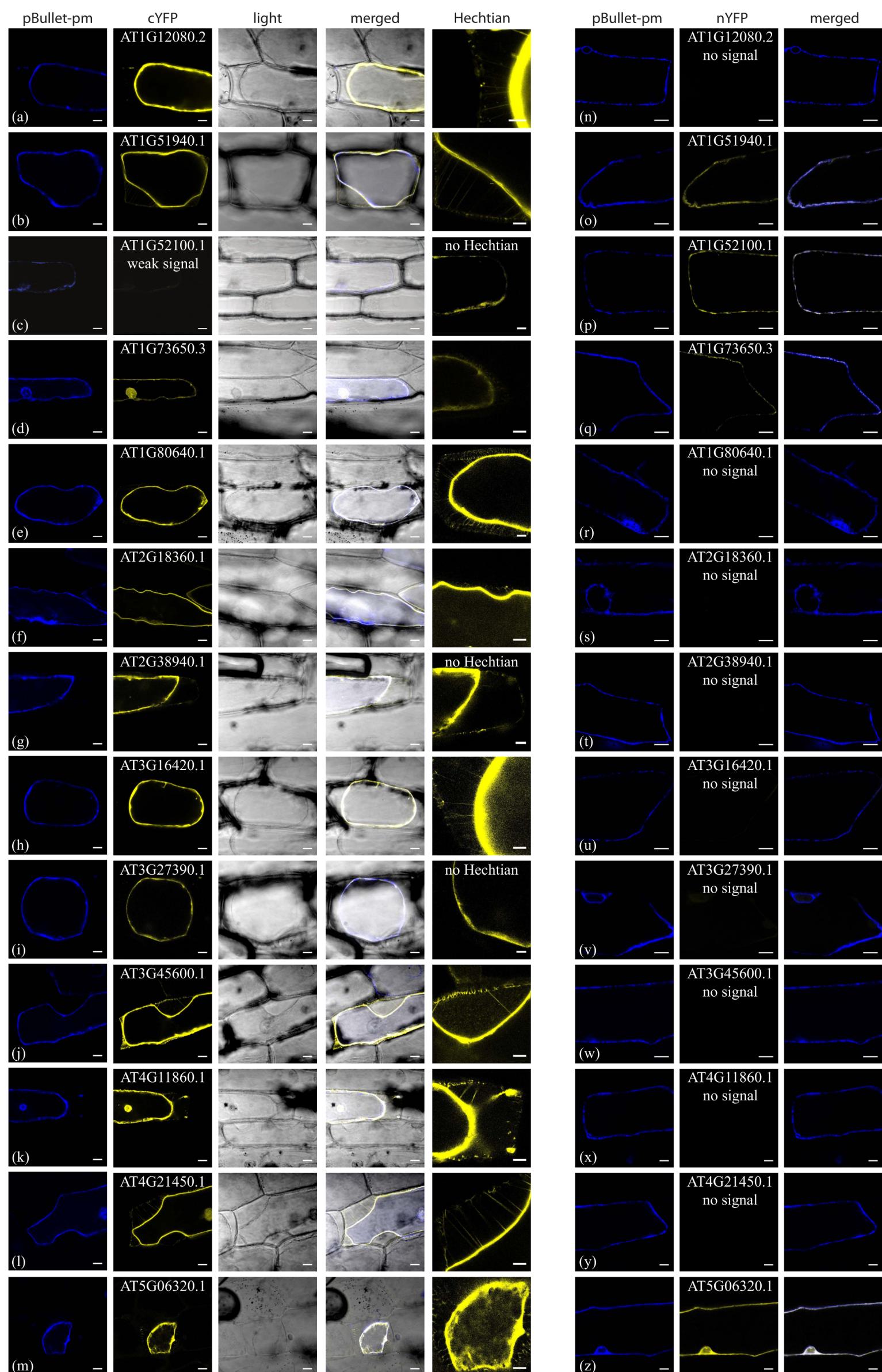
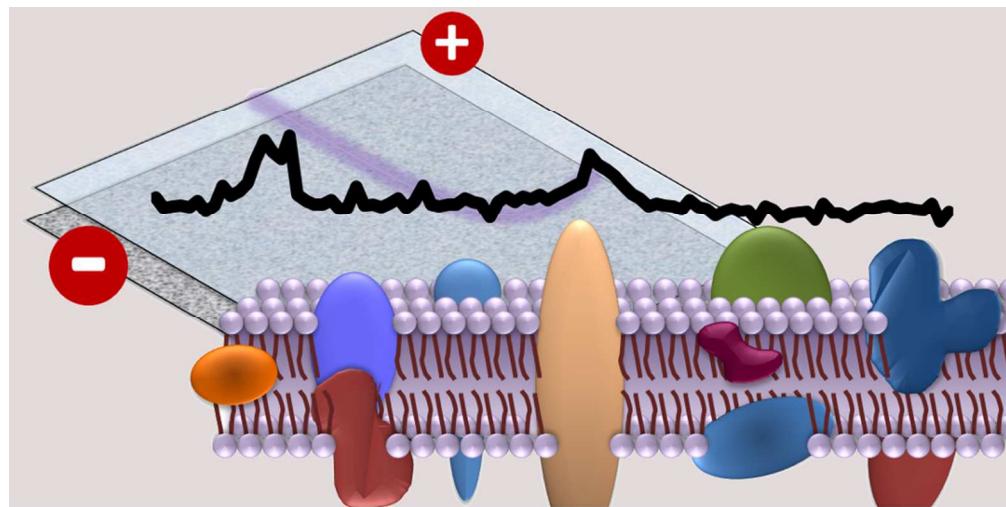


Figure 4



For TOC only

For TOC only
88x55mm (300 x 300 DPI)