A Quantitative Analysis of *Arabidopsis* Plasma Membrane Using Trypsin-catalyzed 18O Labeling*

Clark J. Nelson‡, Adrian D. Hegeman‡, Amy C. Harms‡, and Michael R. Sussman‡§¶

Typical mass spectrometry-based protein lists from purified fractions are confounded by the absence of tools for evaluating contaminants. In this report, we compare the results of a standard survey experiment using an ion trap mass spectrometer with those obtained using dual isotope labeling and a Q-TOF mass spectrometer to quantify the degree of enrichment of proteins in purified subcellular fractions of *Arabidopsis* plasma membrane. Incorporation of a stable isotope, either H$_{2}^{18}$O or H$_{2}^{16}$O, during trypsinization allowed relative quantification of the degree of enrichment of proteins within membranes after phase partitioning with polyethylene glycol/dextran mixtures. The ratios allowed the quantification of 174 membrane-associated proteins with 70 showing plasma membrane enrichment equal to or greater than ATP-dependent proton pumps, canonical plasma membrane proteins. Enriched proteins included several hallmark plasma membrane proteins, such as H$^{+}$-ATPases, aquaporins, receptor-like kinases, and various transporters, as well as a number of proteins with unknown functions. Importantly, a comparison of the datasets from a sequencing “survey” analysis using the ion trap mass spectrometer with that from the quantitative dual isotope labeling ratio method indicates that as many as one-fourth of the putative survey identifications are biological contaminants rather than *bona fide* plasma membrane proteins. *Molecular & Cellular Proteomics* 5:1382–1395, 2006.

The use of tandem MS for the identification of proteins within organelles and subcellular compartments has expanded rapidly in recent years (1–14). This technology reduces the problems associated with a wide dynamic range of protein abundances while also making it easier to draw conclusions regarding biological significance. Large lists of proteins identified via sequence are often generated from such studies, but it is difficult to assign observations as legitimate elements of a specific organelle rather than as contaminants. Even if an observation is *bona fide*, it is unclear whether it is exclusively located in the given compartment or present in many compartments within the cell. Application of some form of quantitation across purification schemes is clearly necessary to increase confidence in the organellar protein assignments.

Most prior studies with plasma membranes purified from *Arabidopsis* have relied on two-dimensional gel electrophoresis for separation and quantification of proteins (1–5). Although capable of separating several hundred proteins at once, two-dimensional gel electrophoresis has significant limitations, particularly with hydrophobic and basic proteins (3, 15). Difficulties in resolving and identifying the various isoforms of plasma membrane integral proteins such as plasma membrane intrinsic proteins (PIPs)$^{1}$ and *Arabidopsis* H$^{+}$-ATPases (AHAs), which are both very hydrophobic multitransmembrane domain-containing polypeptide families, serve as a primary example of this limitation (2, 4, 5). An alternative approach has avoided the problems associated with isoelectric focusing of polytopic membrane proteins by using one-dimensional SDS-PAGE for their protein separations (6, 7). Although successfully identifying hundreds of proteins, including several hydrophobic integral proteins, these studies only validated the degree of purity in their preparations using enzyme assays or Western blots for a handful of marker proteins. An obvious weakness with this method is the contamination issue discussed previously. As an alternative to gel-based methods, shotgun proteomics uses LC for fractionation. In this method, peptides from tryptic digests are fractionated using strong cation exchange (SCX) LC and then subjected to reverse-phase LC prior to analysis via MS. This two-dimensional LC method has proven quite effective, producing thousands of protein identifications in a single analysis (16, 17). One study compared such an off-line 2D LC fractionation scheme with an SDS-PAGE separation approach using chloroplast proteins from *Arabidopsis*. In this comparison, 283 proteins were identified by the 2D LC approach, whereas

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$^{1}$ The abbreviations used are: PIP, plasma membrane intrinsic protein; AHA, *Arabidopsis* H$^{+}$-ATPase; FP, false positive; LOPIT, localization of organelle proteins by isotope tagging; RLK, receptor-like kinase; SCX, strong cation exchange; vATPase, vacuolar type H$^{+}$-ATPase; 2D, two-dimensional; ER, endoplasmic reticulum; TAIR, The *Arabidopsis* Information Resource; Pase, pyrophosphatase; GPI, glycosylphosphatidylinositol; DREPP, developmentally regulated plasma membrane polypeptide; CPK, calcium-dependent protein kinase; PLC, phospholipase C; PLD, phospholipase D.

From the ‡Biotechnology Center and §Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706
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243 were identified by the gel-based approach further validating this approach as a legitimate separation technique (13).

Stable isotopic labeling of peptides or proteins in conjunction with MS analysis is an attractive method for protein quantitation and, as we will demonstrate, provides a facile means for identifying contaminants. A familiar version of this strategy is the commercially available ICAT reagent (18). In one recent organelar proteomic investigation, Dunkley et al. (14) applied this technique to fractions generated by density centrifugation of Arabidopsis total membranes. They labeled adjacent fractions across the density gradient, developing a series of ratios for identified proteins. By then applying multivariate analysis techniques and comparing marker proteins with unknown proteins they identified several novel Golgi and endoplasmic reticulum (ER) components. Dunkley et al. (14) referred to this method as localization of organelle proteins by isotope tagging (LOPIT). A similar strategy using the ICAT reagent was applied in the validation of mitochondrial protein identifications in rat liver (19).

Although successful with some proteins, the ICAT reagent is limited to proteins containing cysteines. Many proteins have few if any cysteines, and their tryptic fragments may not be of the proper size for mass spectral analysis. An alternative labeling strategy uses serine proteases such as trypsin to incorporate two 18O atoms into the carboxyl termini of cleaved peptides (20–26). There are two significant advantages to this strategy. First, it is highly specific with 18O incorporation occurring only at the carboxyl terminus of peptides minimizing spectral complexity and allowing easier and more confident database searches. Second, using trypsin this exchange is nearly global in that all cleaved peptides are labeled except peptides from the carboxyl terminus of proteins that do not terminate with a lysine or arginine.

Here we report the use of 2D HPLC ESI-MS/MS on an ion trap mass spectrometer to identify 309 proteins from a plasma membrane-enriched sample, the largest survey to date. Using trypsin-catalyzed 18O isotopic labeling and 2D HPLC ESI-MSMS on a Q-TOF mass spectrometer for relative quantitation, proteins in a plasma membrane fraction were quantified with 70 proteins showing significant enrichment. A comparison of the two datasets shows that one-sixth to one-fourth of the plasma membrane protein survey data have different ratios and thus represent biological contaminants. Consistent with the role of the plasma membrane in transport and signal transduction, gene ontology predictions indicated that transporters and protein kinases were two of the largest functional categories of sequenced proteins with bona fide plasma membrane origin.

**EXPERIMENTAL PROCEDURES**

**Materials**—All reagents were purchased from Sigma/Aldrich unless otherwise noted.

**Sample Preparation**—Arabidopsis thaliana ecotype Columbia was grown at 22 °C in 24-h light in liquid culture consisting of 0.5% (w/v) MES (pH 5.7), 2.15% (w/v) Murashige and Skoog salts, and 1% (w/v) sucrose. At 2 weeks of age, whole seedlings were harvested. Unless otherwise noted, all subsequent steps were performed at 4 °C in a cold room or on ice. Tissue was weighed and then suspended in ice-cold homogenization buffer (300 μm sucrose, 100 mM Tris (pH 7.6), 25 mM EDTA, 25 mM NaF, 1 mM Na2MoO4, 0.5% (w/v) polyvinylpyrrolidone, 1 mM PMSF, 1 μg/ml pepstatin, 1 μg/ml E-64, 1 μg/ml bestatin, 100 μM 1,10-phenanthroline, and 1 mM DTT) at 1 g of tissue/2 ml of homogenization buffer. The suspension was ground three times in a commercial kitchen style blender for 20 s, filtered through two layers of Miracloth, and subjected to centrifugation at 5,000 × g for 5 min. The supernatant was centrifuged at 80,000 × g for 40 min, and the pellet was subjected to two-phase partitioning using 6.2% (w/w) polyethylene glycol 3350 (Sigma) and dextran (Amersham Biosciences), 4 mM KCl, 5 mM K2HPO4/KH2PO4 (pH 7.8), 1 mM DTT, and 0.1 mM EDTA as described previously (27). The upper phase was diluted 4-fold in resuspension buffer (300 μm sucrose, 10 mM Tris (pH 7.5), and 1 mM EDTA), whereas the lower phase was diluted 1:1 in the same buffer. The membranes were collected by ultracentrifugation at 100,000 × g for 1 h, pellets were washed one more time prior to final resuspension in ~1 ml of resuspension buffer, and the protein content was determined by BCA assay (Pierce).

**Immunodetection and Enzyme Assays**—Following protein quantification, 15 μg of upper and lower phase proteins were separated via SDS-PAGE on 10% Tris-HCl Criterion gels (Bio-Rad) in 2% (w/v) SDS, 63 mM Tris (pH 6.8), 0.01% (w/v) bromphenol blue, 20% (w/v) glycerol, and 100 mM DTT. Following electrophoresis, proteins were electrotransferred onto PVDF-Plus membranes (Osmonics) in transfer buffer (20% (v/v) methanol, 39 mM glycine, and 48 mM Tris). After rinsing in TBST (25 mM Tris (pH 8.0), 140 mM NaCl, 3 mM KCl, and 0.05% (w/v) Tween 20), the membranes were then blocked overnight at 4 °C in Blotto (2% (w/v) defatted milk protein in TBST).

The endoplasmic reticulum marker (Sec12 antigen) antibody was diluted 2000:1 in Blotto (28). The plasma membrane marker (AHA) was diluted 10,000:1 in Blotto (29). Following incubation with primary antibodies, the blots were rinsed with TBST and then incubated with secondary horseradish peroxidase-conjugated antibodies (Kirkegaard and Perry Laboratories) diluted 5000:1 in TBST. Samples were imaged using chemiluminescence (Upstate Cell Systems or Amersham Biosciences). The plasma membrane and endomembrane fractions were also subjected to tandem-sensitive ATPase assays to quantify enrichment for plasma membrane proton pumps as described previously (30).

**Membrane Digest**—For relative quantitation of biological samples, 1.2 mg of protein from the upper and lower phases were incubated in 100 mM NaCO3 (pH 11) at 4 °C for 1.5 h and then pelleted in a microcentrifuge (31). Pellets were resuspended in 600 μl of 50 mM Tris (pH 8.0), 10 mM CaCl2, and 10 mM NaCl. The resuspensions were thermally denatured for 10 min in boiling water, cooled followed by the addition of DTT to a final concentration of 5 mM, and lyophilized in a rotary evaporator SpeedVac (Savant). After lyophilization, samples were resuspended in 300 μl of dry methanol (Acros) using a sonicating bath. This was followed by the addition of 285 μl of natural abundance (0.2% 18O) double distilled H2O or 99% 18O-enriched water (Isotec), and 15 μg of lyophilized sequencing grade modified trypsin (Promega) (resuspended in the appropriate water) was added at 1 μg/μl. The final composition of the solution was 50% (v/v) methanol, 10 mM Tris (pH 8.0), 10 mM CaCl2, 10 mM NaCl, and 5 mM DTT. The digests were allowed to proceed for 12 h at 37 °C, and an additional 15 μg of trypsin were added. After allowing the digest to proceed overnight, the reactions were clarified by centrifugation in a microcentrifuge, and the supernatant was removed. The reactions were then terminated by addition of formic acid to 5% (v/v) of the original volume, and the reciprocally labeled samples were combined and diluted 6-fold. Samples were then desalted via solid-phase ex-
traction with a Spec Plus™ PT400 C_18 cartridge (Ansys) and eluted using 70% (v/v) acetonitrile and 0.1% formic acid (v/v). The peptides were resuspended in 25% (v/v) acetonitrile, 5 mM DTT, and 0.1% formic acid (v/v). Digests used in the ion trap plasma membrane surveys were conducted as for dual isotope-labeled quantitation except that 750 μg of upper phase protein were used and not combined with lower phase digest.

**Off-line SCX Fractionation**—Samples were loaded onto a 150 × 1-mm column home-packed with polySULFOETHYL A™ SCX resin and run using an Alliance HT HPLC system (Waters) at 50 μl/min in buffer A (25% (v/v) acetonitrile and 0.1% (v/v) formic acid). After loading, the following gradient was conducted at 50 μl/min: 0–25% buffer B (25% (v/v) acetonitrile, 1 M NaCl, and 0.1% (v/v) formic acid) over 25 min followed by 25–100% buffer B over 5 min; and fractions were collected every minute. The organic solvents from each fraction were then removed using vacuum centrifugation, and the samples were desalted using C_{18} solid-phase extraction ZipTips (Millipore). Samples were eluted using 70% (v/v) acetonitrile and 0.1% (v/v) formic acid, and the solvent again was removed using rotary evaporation. Samples were then resuspended to ~40 μl in 0.1% (v/v) formic acid and 2% (v/v) acetonitrile and analyzed by LC-MS. Fractions were selected for further analysis based on their absorbance at 215 nm during the SCX separation.

**LC-MS Analysis**—Isotopically labeled samples were analyzed on a Q-TOF 2 mass spectrometer (Micromass) coupled to an HP 1100 HPLC system (Agilent). Analyses were conducted on home-pulled fused silica columns (100 μm × 11 cm) packed with Eclipse C_{18} resin (Agilent). Samples were analyzed using reverse-phase chromatography at 300–500 nl/min with buffer A containing 0.1% (v/v) formic acid and buffer B containing 95% (v/v) acetonitrile and 0.1% (v/v) formic acid. After loading samples in 2% buffer B, the gradient consisted of 2–12% buffer B over 10 min, 12–50% buffer B over 105 min, 50–60% buffer B over 5 min, and 60–100% buffer B over 5 min. The instrument was operated in data-dependent mode with an MS scan followed by a 4-s MS/MS sequencing attempt for the most intense MS peak. Ions within 1.2 Da of the sequenced peak were dynamically excluded for 120 s following a sequencing attempt.

For survey samples, MS analysis was performed with an Agilent 1100 series LC/MSD ion trap mass spectrometer. Samples were loaded using an Agilent 1100 series capillary HPLC system onto a C_{18} reverse-phase trap cartridge (Agilent) and washed for 20 min. Following the loading, the trap column was switched in line with an analytical 75-μm × 150-mm column packed with 3.5-μm Zorbax C_{18} reverse-phase resin. Peptides were eluted from the trap column and further resolved on the analytical column using the following gradient: 5–60% mobile phase B over 60 min, 60–100% mobile phase B over 5 min, held at 100% mobile phase B for 5 min, 100–5% mobile phase B over 5 min, and then held at 5% mobile phase B for 15 min. Mobile phase A consisted of 0.1% (v/v) formic acid, and mobile phase B consisted of 95% (v/v) acetonitrile and 0.1% (v/v) formic acid. During the gradient an MS survey scan was conducted followed by MS/MS sequencing of the five most intense peaks with dynamic exclusion for the gradient an MS survey scan was conducted followed by MS/MS sequencing. For both datasets, peptides scoring above the 1% FP threshold were considered. The high score for peptides unique to a locus in the genome were summed, and all proteins were accepted if they possessed two or more peptides or a single peptide scoring 60 or greater.

Information regarding all identified peptides was extracted from the Mascot search results using Perl scripts that utilized the Msparser method similar to that reported by MacCoss et al. (35). Using this method, the slope of these regressions represented values for the single and double incorporation peaks that were normalized to the monoisotopic peak where the monoisotopic peak had a value of one. A significant benefit of this strategy is that these values are background-subtracted (35). In addition, the correlation coefficient of this strategy provides an estimate of the quality of the fit and can be used as a filter to remove data with poor signal to noise or that have coeluting contaminants (35). For observations with an R^2 value of 0.8 or greater for both regressions, their normalized intensities could then be used to calculate a heavy to light ratio using the equation below that is similar to a method described previously (20).

\[
H/L = \frac{(P_4 - R_2 \times P_0)}{(P_4 - R_2 \times P_0 - (P_2 - R_1 \times P_3) \times R_0)} / P_0 \quad \text{(Eq. 1)}
\]

In this equation, P_0, P_2, and P_4 represent the measured intensities for isotopes of the zero, one, and two heavy oxygen incorporation events (monoisotopic, +2, and +4 isotopic peaks), respectively. The values R_1 and R_0 correspond to the calculated isotopic ratio between the monoisotopic peak and the second and fourth isotopes, respectively, for each peptide based on known natural isotopic abundance using multinomial expansions. On occasion, intense ions with large m/z...
values had multiple isotopes that were smaller than predicted based on natural abundance due to a nonlinear response in the mass spectrometer detector. In those infrequent cases where the second isotope was smaller than predicted, a correction was performed whereby the single $^{18}$O incorporation value was set to zero. If the fourth isotope measurement was also lower than predicted by natural abundance, the peptide was not used in further calculations.

After successful identifications were quantified, ratios were used to calculate an enrichment value for each peptide. Peptides that were unique to a given locus in the genome were then used to calculate an isotopic ratio for the protein. Proteins with three or more peptides were subjected to Dixon’s Q test prior to the calculations (35, 36).

**Informatic Analysis**—The ARAMEMNON web server (aramemnon.botanik.uni-koeln.de) consensus sequence was used for transmembrane domain predictions (37). For subcellular localization, predictions were downloaded from The Arabidopsis Information Resource (TAIR) website (www.arabidopsis.org) and were based on the Pfam algorithm (38). Predictions for amino-terminal myristoylation were also downloaded from the TAIR website. Microarray experiments were queried at the Arabidopsis Membrane Protein Library website (www.cbs.umn.edu/arabidopsis/).

**RESULTS**

Although the ion trap does not have sufficient resolution to quantify small isotopic shifts such as from $^{18}$O labeling compared with a Q-TOF mass spectrometer, it has a shorter duty cycle and is therefore capable of sequencing a larger number of peptides in a typical LC analysis. To assess sample complexity and identify as many potential plasma membrane residents as possible, we first conducted three independent 2D LC analyses of a plasma membrane-enriched fraction using an ion trap mass spectrometer. To quantify plasma membrane enrichment, we applied an $^{18}$O labeling strategy to the upper ion trap mass spectrometer. In those infrequent cases where the second isotope was smaller than predicted, a correction was performed whereby the single $^{18}$O incorporation value was set to zero. If the fourth isotope measurement was also lower than predicted by natural abundance, the peptide was not used in further calculations.

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**False Positives**—Multiple studies have documented the variability of the ability of MS/MS search engines to accurately search through data minimizing both false positives and false negatives (16, 33, 39). False positive rates are found to be a function of several variables including sample complexity, sample handling, charge state, MS platform, database size, and the MS/MS search program used. Although some search engines such as Mascot attempt to provide a probabilistic estimate of peptide identification, it is not surprising that such search engines perform less than perfectly (39, 40). An empirical approach that has proven effective is a reverse database strategy. In this strategy, MS/MS spectra are compared with the database of interest, known as the forward database, as well as with a reverse database. The reverse database contains every protein sequence from the forward database with its sequence reversed. Because the databases are of the same size and amino acid content, it is expected that the number of “chance” hits will be similar in the forward and reverse databases (16, 33).

Because the ion trap was operated in a mode where a preference was set for doubly charged peptides, few singly charged peptides were observed. As a result, the 1% FP cutoff Mascot scores were calculated only for doubly and triply charged peptides (Fig. 2A). The doubly and triply charged peptides had 1% cutoffs of 29 and 39, respectively, for Mascot. For Q-TOF data, the 1% FP thresholds are presented in Fig. 2B. The singly charged peptides had a threshold of 37, whereas doubly and triply charged peptides had a threshold of 21 and 18, respectively.

**MS Surveys**—Samples were digested in aqueous/methanol solutions, and resulting peptides were separated via SCX. Each analysis was processed separately by the criteria described above. After combining the results from the protein identifications, there were 309 protein identifications made by 1016 peptides unique to one locus in the genome. This list of peptides is provided in Supplemental Table 1. Of the 309 proteins identifications, 92 were single peptide identifications. For proteins identified by only one peptide, annotated MS/MS spectra are provided in Supplemental Fig. 1. Of the 309 identifications, 139 were observed in all three analyses, whereas 205 were observed in two or more analyses (Fig. 3A). To the best of our knowledge, this is the largest survey to date of Arabidopsis plasma membrane.

Many of the observed proteins were expected plasma membrane residents such as AHAs, PIPs, and receptor-like kinases (RLKs). Several other proteins of interest such as non-RLKs, G-proteins subunits, and novel proteins were also
identified. However, among these observations were other identified proteins that would not be expected to co-purify with plasma membranes. Some of these unexpected identifications included isoforms of cytochrome $b_5$, porins, ribosomal proteins, proton pyrophosphatase (PPase), tonoplast integral proteins, and vacuolar type H$^+$/H$_{10}$-ATPase (vATPase) subunits. These may represent ER, mitochondrial, soluble, and vacuolar contaminants and have been observed in various plasma membrane survey studies (4–7). Whether these proteins are plasma membrane-localized or simply represent hyperabundant species from other membranes that are contaminants is not readily apparent from simple surveys.

**Isotopic Labeling**—In the first isotopic labeling experiment, the plasma membrane-containing phase was digested in $^{18}$O-enriched water, whereas the endomembrane fraction was digested in natural abundance water. The labels were reversed in the second experiment. From here forward in the text, all reported ratios are normalized so that upper phase-enriched proteins are always shown as values greater than one.

Using the criteria described above for protein identification, there were 116 proteins quantified in the first experiment and 139 in the second experiment. Peptides from the experiments are reported in Supplemental Table 2. Between the two experiments, 174 proteins were quantified. Of these 174 proteins, 38 were identified by the sequencing of only one peptide. An example MS/MS spectrum for a peptide used to describe a single peptide identification is provided in Fig. 4. The MS/MS spectra for the remaining proteins identified by a single peptide are provided in Supplemental Fig. 2. Although there is a large overlap between the survey dataset and the isotopically labeled datasets, there are also significant numbers of protein identifications unique to each dataset. A comparison of overlap between the ion trap and two isotopic labeling experiments is provided in Fig. 3B.

In either of the isotopic labeling experiments, proteins showing enrichment equal to or greater than an AHA were classified as plasma membrane-enriched. By considering each experiment independently, biases resulting from the labeling method were avoided. In total, 70 proteins showed plasma membrane enrichment. Those characterized by multiple peptides are presented in Table I, whereas proteins
described by only one peptide are presented in Table II. A log<sub>2</sub> transform of all proteins quantified in the first dataset is provided as an example of the protein distributions (Fig. 5). As a reference with which to compare isotopic measurements, vanadate-sensitive ATPase assay results, which are used as a measure for *Arabidopsis* H<sup>+</sup>-ATPase abundance, are reported in Fig. 6A for survey data as well as both isotopic labeling experiments.

In a recent study using ICAT for quantification of peptides from microsomal proteins of *Arabidopsis*, 170 proteins were quantified (14). Another study also used the ICAT reagent to quantify 169 proteins demonstrating that 79 were legitimate mitochondrial proteins (19). Based on these other numbers versus our own, 18O labeling performed similarly and is a legitimate alternative as a stable isotope labeling strategy.

From the 70 proteins that showed plasma membrane enrichment, 36 are predicted to possess one or more transmembrane domains. Many of these are considered canonical plasma membrane identifications as determined by alternative methods for localization including microscopic histochemical or reporter gene measurements. In addition, another 12 proteins are predicted to possess a GPI anchor that would confine them to a membrane. One other protein is predicted to possess an amino-terminal myristoylation that would also facilitate membrane localization. Finally four other proteins are predicted to either possess one transmembrane domain or alternatively to possess an amino-terminal myristoylation site. Therefore, 53 proteins are likely physically tethered to the membrane representing 76% of the plasma membrane fraction-enriched proteins. The large fraction of hydrophobic protein identified by this method validates the digestion and fractionation scheme used as a legitimate method for analysis of membrane proteins. Many of the remaining proteins, such as two remorin-like proteins, two developmentally regulated plasma membrane polypeptide (DREPP) isoforms, a phospholipase D, phospholipase C, and two quinone reductases, are documented to interact strongly with the plasma membrane (41–46).

Transporters—After assessing molecular functions using the gene ontology tool available at TAIR, transporters formed...
A Quantitative Analysis of *Arabidopsis* Plasma Membrane

| Gene | Annotation | TDs | Sc1 | P1 | Q1 | Ratio 1 | Sc2 | P2 | Q2 | Ratio 2 |
|------|------------|-----|-----|----|----|---------|-----|----|----|--------|
| At1g01620 | PIP1.3 | 6 | 178 | 3 | 3 | 12.13±6.64 | 46 | 5 | 2 | 32.84±33.85 |
| At1g03870 | FLA9 | 5 | 88 | 1 | 1 | 4.17 | 107 | 2 | 1 | 17.69 |
| At1g05150 | EF hand | 0 | 70 | 2 | 1 | 7.25 | 129 | 2 | 2 | 12.63±0.47 |
| At1g19870 | Calmod.-bind | 0 | 88 | 1 | 1 | 4.17 | 107 | 2 | 1 | 17.69 |
| At1g22530 | SEC14 | 0 | 444 | 10 | 6 | 5.35±0.93 | 671 | 13 | 11 | 11.22±2.10 |
| At1g30360 | ERD4 | 9 | 396 | 9 | 2 | 4.85±3.13 | 394 | 10 | 3 | 10.48±2.74 |
| At1g48480 | LRR Kinase | 1 | 0 | 0 | 0 | — | — | — | — | — |
| At1g59870 | PDR8 | 14 | 481 | 4 | 5 | 4.90±0.96 | 380 | 8 | 6 | 10.28±4.48 |
| At1g59960 | Glycos. hyd. 9 | 0 | 0 | 0 | 0 | — | — | — | — | — |
| At1g78900 | vATPase A | 0 | 174 | 4 | 2 | 7.54±7.52 | 164 | 4 | 3 | 2.22±0.83 |
| At2g04780 | FLA7 | 65 | 1 | 1 | 10.59 | 153 | 3 | 2 | 43.00±27.90 |
| At2g18730 | DAG | 0 | — | — | — | — | — | — | — | — |
| At2g19860 | AHA1 | 10 | 471 | 11 | 8 | 13.00±11.85 | 559 | 11 | 7 | 16.39±6.72 |
| At2g20520 | FLA6 | 107 | 2 | 1 | 8.70 | — | — | — | — | — |
| At2g36380 | PDR6 | 13 | — | — | — | — | — | — | — | — |
| At2g37050 | LRR kinase | 1 | 78 | 2 | 1 | 6.37 | — | — | — | — |
| At2g37170 | PIP2.2 | 6 | 207 | 4 | 3 | 17.05±14.60 | 159 | 3 | 2 | 36.54±1.92 |
| At2g37180 | PIP2.3 | 6 | — | — | — | — | — | — | — | — |
| At2g39010 | PIP2.6 | 6 | 111 | 3 | 1 | 1.32 | 170 | 3 | 3 | 11.45±0.83 |
| At2g45470 | FLA8 | 199 | 3 | 2 | 10.63±0.40 | 171 | 2 | 2 | 31.76±13.43 |
| At2g45820 | Remorin | 0 | 312 | 7 | 4 | 4.56±1.35 | 297 | 6 | 5 | 10.92±3.35 |
| At2g4960 | PIP1.2 | 6 | 193 | 3 | 3 | 8.08±5.17 | 266 | 4 | 3 | 18.00±11.15 |
| At3g01290 | Band 7 | 299 | 6 | 4 | 10.47±7.52 | 380 | 8 | 4 | 14.64±5.97 |
| At3g02880 | LRR kinase | 1 | 295 | 5 | 1 | 9.43 | 378 | 5 | 3 | 21.83±18.05 |
| At3g06390 | Integral | 4 | — | — | — | — | — | — | — | — |
| At3g08510 | PLC2 | 0 | 563 | 13 | 6 | 11.92±6.89 | 558 | 11 | 8 | 16.16±11.86 |
| At3g14840 | LRR protein | 1 | 279 | 3 | 2 | 8.31±2.66 | 362 | 8 | 4 | 22.92±19.33 |
| At3g20410 | CPK9 | 1 | — | — | — | — | — | — | — | — |
| At3g51550 | Protein kinase | 1 | 155 | 2 | 2 | 5.57±0.57 | 153 | 4 | 2 | 28.02±11.84 |
| At3g53420 | PIP2.1 | 6 | 169 | 3 | 1 | 6.49 | 161 | 3 | 2 | 15.95±5.42 |
| At3g57530 | CPK32 | 1 | — | — | — | — | — | — | — | — |
| At3g60330 | AHA7 | 10 | — | — | — | — | — | — | — | — |
| At3g61260 | Remorin | 0 | 280 | 4 | 3 | 7.06±1.24 | 158 | 3 | 2 | 41.12±21.62 |
| At3g61430 | PIP1.1 | 6 | 140 | 2 | 2 | 8.72±5.47 | 119 | 2 | 2 | 28.24±7.44 |
| At3g63260 | MKR1 | 0 | — | — | — | — | — | — | — | — |
| At4g00430 | PIP1.4 | 6 | 120 | 2 | 2 | 12.77±1.90 | 143 | 2 | 2 | 26.57±21.32 |
| At4g04720 | CPK21 | 1 | 114 | 2 | 1 | 8.61 | 101 | 3 | 2 | 16.39±0.56 |
| At4g08850 | LRR kinase | 2 | 155 | 3 | 1 | 8.95 | 328 | 7 | 2 | 10.55±6.20 |
| At4g12420 | SKU5 | 159 | 4 | 2 | 10.42±1.62 | 431 | 7 | 2 | 17.52±17.88 |
| At4g20260 | DREPP | 0 | 426 | 7 | 4 | 6.98±4.99 | 368 | 7 | 5 | 13.36±10.56 |
| At4g23400 | PIP1.5 | 6 | 127 | 3 | 2 | 7.30±1.93 | 243 | 4 | 3 | 16.33±10.60 |
| At4g23650 | CPK3 | 1 | — | — | — | — | — | — | — | — |
| At4g26690 | Phosphodiesterase | 1 | — | — | — | — | — | — | — | — |
| At4g27520 | Plastocyanin | 1 | 247 | 5 | 1 | 10.17 | 259 | 5 | 4 | 16.44±6.32 |
| At4g30190 | AHA2 | 10 | 440 | 8 | 5 | 7.65±4.43 | 449 | 9 | 7 | 12.51±4.30 |
| At4g35100 | PIP2.7 | 6 | 122 | 1 | 1 | 6.95 | 201 | 3 | 3 | 35.68±25.85 |
| At4g35790 | PIP | 0 | 208 | 5 | 2 | 8.08±0.89 | 342 | 8 | 1 | 30.88 |
| At4g36750 | Quin. reduc. | 0 | 160 | 3 | 2 | 7.76±0.51 | 412 | 6 | 3 | 14.13±1.40 |
| At4g37070 | Patatin | 0 | 137 | 3 | 1 | 10.46 | 146 | 3 | 1 | 16.95 |
| At5g08080 | SYP132 | 1 | 183 | 3 | 1 | 6.10 | 298 | 5 | 2 | 50.19±30.08 |
| At5g14150 | Expressed | 0 | 0 | 0 | 0 | — | — | — | — | — |
the largest group of enriched plasma membrane proteins (Fig. 7). The largest group of transporters was the PIPs, which are proteins involved in water transport across the membrane. Of the 13 predicted PIPs in the genome, 11 were observed (PIP1.1, PIP1.2, PIP1.3, PIP1.4, PIP1.5, PIP2.1, PIP2.2, PIP2.3, PIP2.4, PIP2.6, and PIP2.7) in the ion trap survey. The same 11 were observed in the Q-TOF analysis, and all showed significant plasma membrane enrichment.

Another group of hallmark plasma membrane transporters are the AHAs of which six isoforms were identified (AHA1, AHA2, AHA3, AHA4, AHA7, and AHA11). Of these, only AHA3 has definitively been proven to possess plasma membrane localization (47). From the isotopic labeling data, AHA1, AHA2, AHA3, AHA7, and AHA11 were quantified, and all showed ratios typical of plasma membrane protein enrichment in the upper phase.

Among quantified proteins, there were two ATP-binding
cassette superfamily transporters (PDR6 and PDR8), and both were plasma membrane-enriched. Besides the two previously described genes, five other ATP-binding cassette superfamily members were described in the ion trap data (ATH1, MDR4, MDR11, MDR17, and WBC12). Multiple sugar transporters were also observed.

**Kinases** — The known role of the plasma membrane in signal transduction is supported by the larger fraction of kinases identified in the survey and quantified samples as compared with the entire genome (Fig. 7). From the ion trap survey, 10% of the identifications were assigned kinase activity, whereas 14% of the plasma membrane-enriched proteins possessed kinase activity. Kinases comprised only 6% of proteins not showing significant plasma membrane enrichment.

RLKs are a large family of Ser/Thr kinases with over 600 members predicted from the *Arabidopsis* genome (48). Although they are believed to be plasma membrane residents, very little is known about the family as a whole. In total, 31 RLKs were identified in the ion trap survey. From these proteins, 12 RLKs were quantified from the Q-TOF data, and eight of these showed plasma membrane enrichment. Of the four remaining RLKs, all showed some enrichment in the

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**A. PM H⁺-ATPase Activity**

| Sample   | Upper | Lower | Fold Enrichment |
|----------|-------|-------|-----------------|
| Sample 1 | 1841 (±85) | 153 (± 52) | 12x |
| Sample 2 | 2236 (± 114) | 168 (± 102) | 13x |
| Sample 3 | 1980 (±100) | 121 (± 50) | 16x |

**B. Western-Blot Analysis**

![Whole Genome and Survey Data](image_url)

**FIG. 7. Assigned molecular functions.** Shown are the assigned gene ontology molecular functions as acquired from the TAIR website (www.arabidopsis.org). Functions are shown for all predicted gene products, proteins identified in the ion trap survey, proteins with plasma membrane enrichment based on isotopic labeling measurements, and proteins not showing significant plasma membrane enrichment.
plasma membrane-enriched upper phase. Multiple isoforms observed here are previously undescribed in recent plasma membrane proteomic surveys (6, 7).

CPKs are a family of kinases requiring calcium for activation and that contain the kinase and calmodulin-like domains within one polypeptide (49). None of the isoforms has a clearly assigned function to date. From this family, three were characterized in the ion trap survey data: CPK3, CPK9, and CPK21. From the isotopic ratio measurements, there were four CPKs identified that showed plasma membrane enrichment (CPK3, CPK9, CPK21, and CPK32). One isoform (CPK5) did not show enrichment consistent with plasma membrane localization but did show noticeable enrichment. Using microscopic observations of proteins attached to green fluorescent protein, prior work found that CPK9 and CPK21 were plasma membrane-localized, whereas CPK3 was reported to be nuclear or cytosolically located (50). Also present in the plasma membrane fraction was MRK1, a member of the Raf subfamily of the mitogen-activated protein kinase kinase kinase (MAPKKK) family with no described function or phenotype.

Proteins with hydrolase activity also made up a large fraction of plasma membrane identifications from both the ion trap survey and upper phase-enriched proteins identified with the Q-TOF mass spectrometer. Among proteins with hydrolase activity, a phospholipase D (PLD) isoform and a phospholipase C (PLC) isoform with prior studies demonstrating the plasma membrane localization of these proteins using immunoblotting (42, 43). The PLD was shown to bind oleic acid and is proposed to play a role in wound response.

**Novel Proteins**—Nearly one-fifth of all *Arabidopsis* genes encode proteins whose sequences provide no clue to their catalytic functions. The novel proteins with unassigned functions are of great interest. Within the ion trap survey identifications, 59 proteins had no assigned molecular function, whereas 11 of the Q-TOF plasma membrane-enriched proteins had no clear role. Among these proteins were two (At5g44610 and At4g20260) DREPP isoforms (44). A prior study found that a DREPP isoform showed temporary up-regulation in response to cold treatment and suggested a role in calcium-mediated cold adaptation (51). Multiple fasciclin-like arabinogalactans were quantified showing enrichment in the plasma membrane. This group of proteins has no assigned molecular function, but in vertebrate systems arabinogalactans play a role in cell adhesion. These proteins have been observed or predicted in prior studies, and their plasma membrane localization is phospholipase C-sensitive (6, 7, 52, 53).

Other proteins were identified with no assigned molecular function. One is an integral protein (At3g06390) that showed 14-fold enrichment in the second isotopic labeling experiment. This protein appears to be largely confined to root tissue based on microarray experiments reported at the *Arabidopsis* Membrane Protein Library. Another one of these proteins is predicted to be plasma membrane-localized by a GPI anchor (At5g14150). Also present were two other proteins with no discernible function or homologs with defined function (At2g38480 and At3g11800). To the best of our knowledge, none of these four proteins has been described in proteomic studies.

Remorins are a group of proteins that have no transmembrane domains or lipid attachment domains but are found to be plasma membrane-localized and -enriched in lipid raft preparations (6, 7, 46). Although two identified remorin-like proteins have putative functions of DNA binding, this is likely not their true role (At2g45820 and At3g61260). First identified as DNA-binding proteins (54), they were later shown to bind other polyanions as well, particularly oligogalacturonides when phosphorylated, with significantly higher affinity than for DNA (45). Additionally microscopy indicates that remorins form filamentous fibers *in vitro* that interact with the plasma membrane in apical regions, particularly root tips (55), but there is no clear biological function for these proteins. Our isotopic measurements clearly establish plasma membrane enrichment for these two proteins.

**Contaminants**—Identification of a protein in plasma membrane-enriched fractions does not necessarily make the protein a *bona fide* plasma membrane identification. This protein may be co-localizing to the plasma membrane or alternatively a hyperabundant protein from a contaminating endomembrane. This is the primary difficulty with organelar proteomics. The isotopic measurements we observed here help to clarify this issue.

Different organelles showed varying levels of depletion from the plasma membrane-enriched phase. The most significant contamination comes from vacuolar components, such as PPases and vATPase subunits, which are considered canonical vacuolar proteins. Vacuolar proteins identified by two or more peptides are presented in Table III. Members of these

| Gene       | Annotation | TDs | Sc 1 | P1 | Q1   | Ratio 1 | Sc 2 | P2 | Q2 | Ratio 2 |
|------------|------------|-----|------|----|------|---------|------|----|----|---------|
| At1g78900  | vATPase A  | 0   | 174  | 4  | 2    | 7.54 ± 7.52 | 164  | 4  | 3  | 2.22 ± 0.83 |
| At1g12840  | vATPase C  | 0   | 109  | 3  | 2    | 0.60 ± 0.07 | 134  | 4  | 3  | 1.38 ± 0.59 |
| At4g11150  | vATPase E  | 0   | 151  | 3  | 2    | 1.72 ± 0.36 | 172  | 3  | 2  | 3.26 ± 0.84 |
| At3g42050  | vATPase H  | 0   | 115  | 2  | 1    | 2.24    | 118  | 3  | 1  | 2.03    |
| At4g39080  | vATPase    | 6   | 120  | 3  | 1    | 0.92    | 83   | 2  | 2  | 2.00 ± 0.65 |
| At1g15690  | PPase      | 14  | 260  | 5  | 3    | 0.39 ± 0.08 | 102  | 3  | 2  | 1.20 ± 0.18 |
three groups have been observed in multiple plasma membrane investigations (3, 6, 7) and have also been reported in detergent-resistant membrane preparations from Arabidopsis and tobacco (8, 46). Using immunological techniques, Robinson et al. (56) detected these proteins at the plasma membrane in pea as well. Most of these proteins typically showed 2-3-fold enrichment in the upper phase in our experiments.

There were multiple proteins observed that are likely to be of ER origin identified by multiple peptides (Table IV). Among the identifications were three isoforms of cytochrome \(b_6\). We also observed a protein called “shepherd” (SHD), which is a 90-kDa heat shock protein with ER localization that assists in the folding of the secreted peptide hormone clavata (57). In addition to these proteins, non-unique peptides representing multiple isoforms of luminal binding protein, an ER chaperone protein, showed similar levels of enrichment (data not shown). Although these proteins seem likely to be contaminants, other reports in the literature suggest that the issue is more complex. Using an \(^{35}S\)-driven transient expression system, Marmagne et al. (7) reported plasma membrane localization for the same cytochrome \(b_6\) (At5g53650) observed here. In this study, all cytochrome \(b_6\) isoforms showed levels of contamination similar to other ER proteins suggesting that these proteins are not plasma membrane-localized. As a comparison, a reference Western blot analysis was performed for Sec12, a marker for ER (Fig. 6B).

Multiple mitochondrial proteins were also observed in this study with most showing 5-10-fold depletion from the upper phase. Some example mitochondrial proteins are presented in Table V. On this list were multiple chloroplast proteins identified by multiple peptides including multiple chlorophyll-binding proteins and a ribulose-bisphosphate carboxylase/oxygenase activase. Most of these proteins showed 10-fold or greater depletion from the plasma membrane-containing upper phase. Several of these proteins are provided in Table VI.

### DISCUSSION

Early proteomic investigations of Arabidopsis had difficulties identifying integral proteins due to the hydrophobicity of these proteins (1, 2, 4, 5). More recent surveys fractionated proteins using SDS-PAGE, successfully identifying many integral membrane proteins (6, 7). However, these studies were non-quantitative in nature, relying on enzyme assays and Western blots to validate their sample preparation. Although many of the observations from these investigations were legitimate and characteristic of bona fide plasma membrane...
localization, it is impossible to rigorously exclude contaminants from such protein identification catalogues. Using isotopic labeling, we have begun to address the issue of contamination. In our study, vascular contaminants showed the largest degree of contamination with several canonical vascular proteins showing mild enrichment in the plasma membrane, whereas others showed little or no depletion from the upper phase. Besides vascular contamination, multiple canonical ER-localized proteins also showed poor depletion from the plasma membrane-enriched phase as well as several cytosolic proteins. The mitochondrial and chloroplast proteins showed more significant depletion relative to these other membrane systems.

Overall the comparison of our isotope ratio measurements with a non-quantitative “survey” study performed side by side indicates that as many as one in four proteins identified by simply sequencing proteins within a plasma membrane-enriched fraction are in fact contaminants rather than bona fide plasma membrane proteins. If one considers proteins contained in all three surveys with quantified proteins, 16% of the plasma membrane proteins. If one considers proteins contained in all three surveys with quantified proteins, 16% of the proteins showed little or no depletion from the upper phase. Besides technical variability, possible biological factors contributing to the range of enrichment values include the following. 1) Plasma membranes isolated from different cell types may have sufficiently different biophysical properties (e.g. different lipid/protein ratios or different surface charges) to preclude identical partitioning in the two polyethylene glycol/dextran phases. 2) Some proteins co-localize to plasma membrane as well as other membrane systems. 3) There may be some retention of plasma membrane proteins within endomembranes due to dynamic fluxes during vesicle trafficking. Although these possibilities may be difficult to quantify or detect in microscopic observations, they would lower the ratio observed in our isotopic measurements. In any case, it is clear that more rigorous quantitative methods for organellar proteomics provide a better framework for interpreting the biological function of proteins and the intracellular compartments in which they exist.

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A Quantitative Analysis of Arabidopsis Plasma Membrane

To whom correspondence should be addressed: Biotechnology Center, University of Wisconsin, 425 Henry Mall, Madison, WI 53706. Tel.: 608-262-8608; Fax: 608-262-6748; E-mail: mssusman@wisc.edu.

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