Identification of Regulatory and Cargo Proteins of Endosomal and Secretory Pathways in Arabidopsis thaliana by Proteomic Dissection*Ś

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The cell’s endomembranes comprise an intricate, highly dynamic and well-organized system. In plants, the proteins that regulate function of the various endomembrane compartments and their cargo remain largely unknown. Our aim was to dissect subcellular trafficking routes by enriching for partially overlapping subpopulations of endosomal proteomes associated with endomembrane markers. We selected RABD2a/ARA5, RABF2b/ARA7, RABF1/ARA6, and RABG3f as markers for combinations of the Golgi, trans-Golgi network (TGN), early endosomes (EE), secretory vesicles, late endosomes (LE), multivesicular bodies (MVB), and the tonoplast. As comparisons we used Golgi transport 1 (GOT1), which localizes to the Golgi, clathrin light chain 2 (CLC2) labeling clathrin-coated vesicles and pits and the vesicle-associated membrane protein 711 (VAMP711) present at the tonoplast. We developed an easy-to-use method by refining published protocols based on affinity purification of fluorescent fusion constructs to these seven subcellular marker proteins in Arabidopsis thaliana seedlings. We present a total of 433 proteins, only five of which were shared among all enrichments, while many proteins were common between endomembrane compartments of the same trafficking route. Approximately half, 251 proteins, were assigned to one enrichment only. Our dataset contains known regulators of endosome functions including small GTPases, SNAREs, and tethering complexes. We identify known cargo proteins such as PIN3, PEN3, CESA, and the recently defined TPLATE complex. The subcellular localization of two GTPase regulators predicted from our enrichments was validated using live-cell imaging. This is the first proteomic dataset to discriminate between such highly overlapping endomembrane compartments in plants and can be used as a general proteomic resource to predict the localization of proteins and identify the components of regulatory complexes and provides a useful tool for the identification of new protein markers of the endomembrane system. Molecular & Cellular Proteomics 14: 10.1074/mcp.M115.050286, 1796–1813, 2015.

Membrane compartmentalization is an essential mechanism for eukaryotic life, by which cells separate and control biological processes. Plant growth, development, and adaptation to biotic and abiotic stress all rely on the highly dynamic endomembrane system, yet we know comparatively little about the proteins regulating these dynamic trafficking events. The plasma membrane (PM) provides the main interface between the cell and its environment, mediating the transfer of material to and from the cell and is a primary site for perception of external signals. Transmembrane proteins are synthesized in the endoplasmic reticulum (ER) and trafficked to the PM via the Golgi, although there are other secretory routes for soluble cargo (discussed in (1–4)). Post-Golgi trafficking is the main route by which newly synthesized transmembrane proteins and cell wall glycans are delivered to the PM. In plants, secretory and endocytic traffic converge at the trans-Golgi network (TGN), which also functions as an early endosome (EE). Multivesicular bodies (MVBs) are the other main endosomal compartment in plants and serve as pre-vacuolar compartments (PVCs) or late endosomes (LE) destined for vacuolar degradation (reviewed (1, 5, 6)).

Recycling and sorting of plasma membrane proteins is essential for generating the polar localization of auxin efflux transporters (discussed in (7)), formation of the cell plate during cell division (8–11), and in defense such as localized deposition of papilla reviewed in (12, 13). Furthermore, the subcellular localization of transporters and receptors is dynamically regulated. For example, the boron transporter (BOR1) exhibits polar localization and is internalized and degraded under conditions of high boron to reduce toxicity (14, 15). Similarly the receptor-like kinases (RLKs) flagellin-sensing 2 (FLS2) and brassinosteroid insensitive 1 (BRI1), important transmembrane receptors in antibacterial immunity and plant development, respectively, are constitutively endocytosed and recycled to the PM (16–18). Both receptors and transporters are also cargoes of the LE/MVB trafficking route (16).
porters (9, 25–27). These include regulators of trafficking that have been developed as useful probes to visualize the different modes of cargo transport. For example, RABD2a/ARA5 labels the Golgi and TGN/EE as well as coated vesicles (25, 29), and RABG3f localizes to MVBs and the tonoplast (26). The VHA-A1 affinity purification data were then further refined using density gradient centrifugation to differentiate cargo and steady-state proteins (45).

We have further explored affinity purification of fluorescent-tagged markers localizing to defined compartments to identify proteins associated with trafficking. Our motivation was to dissect the trafficking routes by enriching for partially overlapping subpopulations of endosomal proteomes associated with small GTPases in the Rab family. We selected RABD2a/ARA5, RABF2b/ARA7, RABF1/ARA6, and RABG3f as markers for the Golgi/TGN/EE/secretory vesicles, LE/MVB compartments, LE/MVB compartments and LE/MVB/tonoplast, respectively. Additionally, we used Golgi transport 1 (GOT1), which localizes to the Golgi, clathrin light chain 2 (CLC2) labeling clathrin-coated vesicles (CCVs) and pits and the vesicle-associated membrane protein 711 (VAMP711) present at the tonoplast (26, 27, 29, 46, 47) as comparisons. Our objective was to identify transient cargo proteins, tethers, and docking factors associated with dynamic subdomains of the endomembrane system, to supplement better-characterized “steady-state” components, and to identify components of recycling and vacuolar trafficking pathways.

**EXPERIMENTAL PROCEDURES**

**Plant Materials**—The following A. thaliana lines were used in this study (accession Columbia-0 if not otherwise stated): (Wave lines 1, 5, 29, 9, and 18) pUB:mCherry and YFP, YFP-RABG3f, YFP-RABD2a/ARA5, YFP-VAMP711, YFP-GOT1 (26), RABF1/ARA6-RFP, RABF2b/RFP-ARA7 (provided by K. Schumacher, Heidelberg, Germany), p35S:CLC2-GFP in W-2 (Wassilewskija) background (provided by S. Bednarek, Madison, WI). For protein extraction and affinity purification, 0.1 g of A. thaliana seed for all constructs were grown in sterile 200 ml of Murashige and Skoog medium at 22 °C, 16 h light, shaken at 120 rpm. For microprojectile bombardment assays, 4–6 weeks old A. thaliana plants were grown on soil under controlled conditions of 22 °C, 12 h light, 60% humidity.

**Protein Extraction and Affinity Purification**—For all constructs, 30–50 g of 8-day-old A. thaliana seedlings were harvested and frozen in liquid nitrogen and ground with a pestle and mortar. Protein extraction buffer (150 mM Na-HEPES (pH 7.5), 10 mM EDTA, 10 mM EGTA, 17.5% (w/v) sucrose, 7.5 mM KCl, 0.01% (v/v) Igepal CA-630, 10 mM dithiothreitol, 1% (v/v) protease inhibitors (Sigma), 0.5% (v/v) polyvinylpyrrolidone) at 2 ml to 1 g of fresh weight tissue was added. All subsequent steps were performed at 4 °C. Protein concentration was determined (usually 0.4–0.6 g total protein) with BCA assay using BSA as the standard. Homogenate was filtered through two layers of miracloth and centrifuged at 6,000 g for 20 min. 20 μl of chromotek GFP or Red Fluorescent Protein (RFP) trap Sepharose beads (as appropriate) were added per 50 ml homogenate and incubated for 3 h with gentle shaking. The homogenate was then centrifuged at 500 g for 5 min and the supernatant discarded. The beads were washed five times with fresh prechilled extraction buffer (no polyvinylpyrrolidone or protease inhibitors) with 3 min incu-
bation. The slurry was collected after the last wash and protein eluted with 2x SDS-PAGE loading buffer and taken for either LC-MS or Western blotting.

**Western Blotting and Immunodetection**—10% polyacrylamide SDS-gels were run at 100/200 V and proteins electroblotted onto PVDF membranes at 250 mA (Bio-Rad). Membranes were rinsed in TBS and blocked in 5% (w/v) nonfat milk powder in TBS 0.1% Tween (TBST) (v/v) for 1 h. Primary antibodies were diluted in 0.5% (w/v) nonfat milk, TBST to the following concentrations and incubated at room temperature for 1 h. Primary antibodies were: anti-AHA1 (H^+ ATPase 1) 1:2,000 (Agrisera AS07 260), anti-BIP2 (luminal binding protein) 1:2,000 (Agrisera AS09 614), anti-RbcL (Rubisco large subunit) 1:10,000 (Agrisera AS03 037), anti-COX2 (cytochrome oxidase 2) 1:5,000 (AS04 053 Agrisera), anti-RFP 1:10,000 (Abcam ab43771). Membranes were washed three times in TBST before 1 h incubation with secondary antibodies anti-rabbit-HRP and anti-mouse-HRP 1:10,000 (Agrisera AS03 037), anti-RFP 1:10,000 (Abcam ab43771). Signals were visualized using chemiluminescent substrate (Lumigen ECL, GE Healthcare) and GE healthcare Image Quant LAS 3000.

**Tryptic Digestion of Proteins**—Affinity purified proteins were separated on 4–20% Tris-glycine nUView precast gradient gels (NuSep). The SDS-PAGE gels were cut into seven slices per affinity purification. Gel slices were washed for 30 min with 50% acetonitrile in 50 mM ammonium bicarbonate (ABC) at 37 °C, twice. Then 100% ACN was added for 10 min and the liquid removed. 10 mM DTT in 50 mM ABC was added to cover the gel pieces for 30 min at 56 °C shaking and the supernatant removed. 55 mM chloroacetamide in ABC (in the dark) was applied for 20 min. The gel pieces were washed twice for 15 min with 50% ACN/25 mM ABC and dehydrated with 100% ACN for 10 min. 1 μg of trypsin, 46 mM ABC, 5% ACN was applied at 37 °C overnight, and the supernatant removed and rehydrated. The gel pieces were washed three times by addition of 50% ACN, 5% formic acid, and sonicated for 10 min, and the wash supernatants were then pooled with previous supernatants. The supernatants containing the peptides were then lyophilized. The control samples, from plant expressing the fluorescent tags mCherry or GFP only, were treated as above except that the trypptic digestions were performed from the affinity beads directly, and these samples were analyzed on an Orbitrap Fusion, not an Orbitrap XL as detailed below.

**Mass Spectrometry**—LC-MS/MS analysis was performed using a hybrid mass spectrometer LTQ-Orbitrap XL (ThermoFisher Scientific) and a nanoflow-UHPLC system (nanoAcquity, Waters Corp.). Peptides eluted in 2% acetonitrile, 0.2% trifluoroacetic acid were applied to a reverse phase trap column (Symmetry C18, 5 μm, 130 C18, 1.7 mm, 75 μm × 250 mm, Waters Corp.) in vented configuration using a nano-T coupling union. Peptides were eluted in a gradient of 3–35% acetonitrile at 0.1% formic acid (solvent B) acid over 90 min followed by gradient of 90% B over 5 min at a flow rate of 300 nL min⁻¹ at 40 °C. The mass spectrometer was operated in positive ion mode with nanoelectrospray ion source with ID 0.02 mm fussed silica emitter (New Objective). Voltage +2.5 kV was applied to the metal-coated tip. Transfer capillary temperature was set to 265 °C, no sheath gas, and the focusing voltages in factory default setting were used. The Orbitrap, MS scan resolution of 120,000 at 400 m/z, range 300 to 1,500 m/z was used, and AGC target was set to 400,000 counts, and maximum inject time to 50 ms. In the dual pressure ion trap, MS/MS spectra were triggered for charge states 2+ to 7+ using Higher-energy collisional dissociation (HCD) fragmentation at 35% and a maximum speed strategy where variable numbers of MS/MS spectra would be acquired within a 2 s window. The threshold for ion selection was 5,000 counts, and selected parent ions were isolated using a mass window of 1.6 in the quadrupole. Fragment ions were detected using the a rapid ion trap scan rate, AGC accumulation target was set to 10,000 counts, and maximum inject time to 200 ms and range 100–2,000 m/z. Dynamic exclusion was enabled allowing for one repeat only, with a 45 s exclusion time, and maximal size of dynamic exclusion list 500 items. Chromatography function to trigger MS/MS event close to the peak summit was used with correlation set to 0.9 and expected peak width 7 s. Charge state screening was enabled enabled for all 2+ charge states and higher to be selected for MS/MS fragmentation. Control samples were analyzed on an Orbitrap Fusion (ThermoScientific) with a Dionex Ultimate 3000 nanoLC system, with a TRAP 50 cm C18 BEH column column form ThermoScientific. Peptides were eluted in a gradient of 3–35% acetonitrile in 0.1% formic acid (solvent B) acid over 90 min followed by gradient of 90% B over 5 min at a flow rate of 300 nL min⁻¹ at 40 °C. The mass spectrometer was operated in positive ion mode with nanoelectrospray ion source with ID 0.02 mm fussed silica emitter (New Objective). Voltage +2.5 kV was applied to the metal-coated tip. Transfer capillary temperature was set to 265 °C, no sheath gas, and the focusing voltages in factory default setting were used. The Orbitrap, MS scan resolution of 120,000 at 400 m/z, range 300 to 1,500 m/z was used, and AGC target was set to 400,000 counts, and maximum inject time to 50 ms. In the dual pressure ion trap, MS/MS spectra were triggered for charge states 2+ to 7+ using Higher-energy collisional dissociation (HCD) fragmentation at 35% and a maximum speed strategy where variable numbers of MS/MS spectra would be acquired within a 2 s window. The threshold for ion selection was 5,000 counts, and selected parent ions were isolated using a mass window of 1.6 in the quadrupole. Fragment ions were detected using the a rapid ion trap scan rate, AGC accumulation target was set to 10,000 counts, and maximum inject time to 200 ms and range 100–2,000 m/z. Dynamic exclusion was enabled allowing for one repeat only, with a 45 s exclusion time, and maximal size of dynamic exclusion list 500 items.

**Software Processing and Peptide Identification**—Peak lists in format of Mascot generic files were prepared from raw data using Proteome Discoverer v1.2 (ThermoFisher Scientific). Peak picking settings were as follows: m/z range set to 300–5,000, minimum number of peaks in a spectrum was set to 1, Signal/Noise threshold for Orbitrap spectra set to 1.5, and automatic treatment of unrecognized charge states was used. Peak lists were searched on Mascot server v.2.4.1 (Matrix Science, London, UK) against the The Arabidopsis Information Resource (TAIR) (version 10) database with GFP, RFP, and common contaminants such as keratin added. Only trypptic peptides were permitted with up to two possible misalignments and charge states +2, +3, +4 were allowed in the search. The following modifications were included in the search: oxidized methionine (variable), carbamidomethylated cysteine (static). Data were searched with a monoisotopic precursor and fragment ions mass tolerance 10 ppm and 0.8 Da, respectively. Mascot results were combined in Scaffold v. 4 (Proteome Software, Portland, OR) and exported in Excel (Microsoft Office). Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Peptide
Affinity Enrichment of Subcellular Proteomes—To identify the proteomes of various endomembrane compartments, we simplified published affinity purification methods for the enrichment of vesicle-associated proteins (44, 45) by removing the sucrose density gradient step and extending the number of selected subcellular markers to create a large-scale, comparative proteomics approach. For this, we used sterile grown *A. thaliana* seedlings stably expressing GOT1, RABD2a/ARA5, RABG3f, and VAMP711 fused to YFP; RABF2b/ARA7 and RABF1/ARA6 fused to the monomeric RFP; CLC2-GFP; and as controls, seedlings expressing the fluorescent proteins only (Fig. 1A, Table I). All these markers were under the control of constitutive promoters ensuring sufficient expression levels and are detected in seedling leaves at their assigned subcellular localizations (Fig. 1B). Total seedlings were harvested after 8 days of hydroponic growth, homogenized, and the proteins extracted in buffer followed by single low speed centrifugation. Using the workflow outlined in Fig. 2A, we affinity purified RFP-RABF2b/ARA7 and YFP-RABG3f from the respective transgenic lines and assessed the enrichment for possible organelle contamination using Western blotting. Our approach greatly enriched the relevant bait proteins with no detectable contamination from the ER, mitochondria, chloroplasts, and PM as revealed by our failure to detect BIP2 (ATSG42020), COXII (ATMG00160), RbcL (ATCG00490), and AHA1 (AT2G18960) with αBIP2, COXII, RbcL, and AHA1 antibodies, respectively (Fig. 2B). No bands to either of RFP, YFP, or the organelle markers were identified in affinity purifications from wild-type seedlings, indicating that abundant cytosolic or membrane-associated proteins did not generally bind to the affinity beads (Fig. 2B). We then proceeded to identify and compare the proteomes of seven different subcellular marker lines. We affinity purified YFP-GOT1, YFP-RABD2a/ARA5, YFP-RABG3f and YFP-VAMP711, RFP-RABF2b/ARA7, RABF1/ARA6-RFP, and CLC2-GFP from the respective transgenic *A. thaliana* seedlings using GFP and RFP beads. We refer to these affinity purifications as “endoosomal-enriched proteomes” and acknowledge that, because we used liquid nitrogen to freeze plant material, it is possible that larger compartments might not remain intact. In this manuscript, we use the term “compartment” as a descriptive term for any membrane-bound structure that is not an organelle, regardless of its size.

Identification and Comparison of Proteins from the Seven Affinity Enrichments—The proteins from each affinity enrichment were separated by one-dimensional gel electrophoresis and identified by mass spectrometry. The full dataset is available from the proteomics database PRIDE (accession numbers PXD001877 to PXD1885 inclusive. Table S1). All affinity enrichments contained peptides from the corresponding bait protein except for YFP-GOT1 that was only identified by peptides from YFP. This is probably because GOT1 is less amenable to tryptic digests, with only two potential peptides between 4 and 24 amino acids in length (Fig. S1). Proteins were accepted as significantly enriched by a specific bait if their SAINTexpress score was 0.8 or higher (49, 50). The SAINT scores and spectrum counts for all proteins assigned to each affinity enrichment, replicate, and associated controls are summarized in Table S2. After SAINT analysis, 433 proteins were accepted being significantly enriched with at least one bait and each affinity enrichment had a number of unique and shared proteins (Table II). Most proteins were identified from the RFP-RABF2b/ARA7 enrichment (279 proteins in total) of which 43% were uniquely assigned to this bait. The CLC2-GFP and YFP-VAMP711 baits had the smallest number of proteins assigned to their enrichments (49 and 51 proteins, respectively) with smaller proportions...
**Endomembrane targets.** (A) Schematic overview of the endomembrane marker proteins used in this study and their localizations. RABD2a/ARA5—post-Golgi/Golgi/TGN/SV, RABF1/ARA6—LE/MVBs, RABF2b/ARA7—LE/MVBs, CLC2—clathrin-coated vesicles, GOT1—Golgi, RABG3f—LE/MVB/vacuole, VAMP711—vacuole. Modified from (16). (B) Localization of fluorescent-tagged marker proteins. Standard confocal micrographs of leaf epidermal cells of A. thaliana transgenic plants stably expressing the indicated recombinant proteins. Scale bars 10 μm. References: pUB:YFP-RabG3f, YFP-RABD2a/ARA5, YFP-VAMP711, YFP-Got1 (26), RABF1/ARA6-RFP, RFP-RABF2b/ARA7 (provided by K. Schumacher, Heidelberg, Germany), p3SS:CLC-GFP (provided by S. Bednarek, Madison WI).
portion of unique proteins assigned to each; only nine (14%) were unique to RABF1/ARA6-RFP while 30 (48%) were unique to YFP-GOT1.

As several of our bait proteins have related biological functions, it is not surprising that we assigned many proteins to more than one affinity enrichment. In addition to excluding proteins that were enriched with the fluorescent protein baits only, our comparison of seven different baits provides a further measure of likely common contaminant proteins. Only five proteins were significantly enriched in all affinity purifications, and four proteins were enriched with six of the seven baits. These include chaperonin 60A and 60B (CPN60; AT1G55490, AT2G28000), the ABC-type transporter PEN3/ABCG36 (AT1G59870), and two ARM repeat family proteins (AT5G53480, AT5G19820). Several chaperones and ribosomal components are considered to be contaminants in affinity purifications, and we identified five heat shock proteins, six additional CPN60/TCP-1 proteins, and four ribosomal proteins with some, but not all, of our baits. The RFP-RABF2b/ARA7 and YFP-RABG3f enrichments contained several of these proteins, and it is possible that the abundance of proteins associated with protein expression and folding are correlated with the expression level of the bait protein. BIP2 (AT5G42020) and COXII (AT1G02410) RbcL (ATCG00490), which we tested for in our Western blot assessment of contamination from organelles, were not significantly enriched with any bait (Fig. 2B). However, two plasma membrane ATPases were identified with specific baits and might represent contamination with plasma membrane proteins or normal secretion, turnover, and recycling of these abundant proteins. AHA1 (AT4G30190) was identified with RFP-RABF2b/ARA7 and AHA2 (AT2G18960) with three baits.

To determine if proteins with known functions in endomembrane trafficking were identified in our affinity enrichments, we manually curated a set of endomembrane regulators from the literature (Table S3) and present the fold enrichment over controls with each of our seven baits (Table III). This set of established endomembrane proteins was differentially enriched with each of the seven baits and largely corresponds to the known biological functions of our bait proteins.

**Table I**

| ATG number | A. thaliana | Mammals | Saccharomyces | Saccharomyces | Expected locations | Construct |
|------------|-------------|---------|---------------|---------------|--------------------|-----------|
| AT3G18820  | RABG3f      | RAB7    | YPT7          | YPT7          | LE/MVB/Ton         | YFP-RabG3f|
| AT4G16640  | ARA7/RABF2b | RAB5    | YPT51/52/53   | YPT5          | LE/MVB             | RFP-RABF2b/ARA7|
| AT3G54840  | ARA6/RABF1  | RAB22   |               |               | LE/MVB             | RABF1/ARA6-RFP|
| AT1G02130  | ARAS/RABD2a | RAB1    | YPT1          | YPT1          | Golgi/TGN/EE/SV    | YFP-RABD2a/ARA5|
| AT4G32150  | VAMP711     | VAMP7   | YKT6          | sec22         | Ton                | YFP-VAMP711|
| AT2G40060  | CLC2        | CLTB    | CLC1          | CLC           | CCV                | CLC2-GFP|
| AT3G03180  | GOT1        | GOT1A   | GOT1p         |               |                    | YFP-GOT1|

**Fig. 2.** Proteomic workflow and validation. (A) Workflow for the affinity enrichment and analysis of endomembrane proteins from *A. thaliana*. (B) Immunoblotting of RFP-RABF2b/ARA7 and YFP-RABG3f proteome enrichments to determine organelle contamination. Total protein extracts, from *A. thaliana* Col-0 or stably expressing RFP-RABF2b/ARA7 or YFP-RABG3f were subjected to immunoblot analysis of membranes or cytosol (Fig. 2B). However, two plasma membrane ATPases were identified with specific baits and might represent contamination with plasma membrane proteins or normal secretion, turnover, and recycling of these abundant proteins. AHA1 (AT4G30190) was identified with RFP-RABF2b/ARA7 and AHA2 (AT2G18960) with three baits.

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To visualize the differences and commonalities in the seven affinity enrichments, we used the Sungear tool from Virtual plant (51) (Fig. 3). On the Sungear plot, the CLC2-GFP and YFP-VAMP711 enrichments appear to be distinct from the other enrichments, but, as discussed above, ~70% of their assigned proteomes are shared with other baits. However, there are few binary overlaps between CLC2-GFP and YFP-VAMP711, rather the shared proteins are common to many baits. The RFP-RABF2b/ARA7 and YFP-RABG3f proteomes have high numbers of uniquely assigned proteins and share a large binary overlap, possibly representing the LE/MVB compartments. There is also substantial pairwise and collective overlaps between the YFP-RABD2a/ARA5, RFP-RABF2b/ARA7, and YFP-RABG3f. The RABF1/ARA6-RFP enrichment has only nine unique proteins and is nearly a subset of RFP-RABF2b/ARA7.

**Our Method Preferentially Enriches Proteins Transiently Associating with Compartments**—To compare the proteins we identified with published proteomic studies, we compiled a comprehensive dataset of proteins identified in the TGN/EE (44), TGN (45), ER, Golgi, mitochondria/plastid, PM, vacuole (39), Golgi (37), ER, Golgi, mitochondria/plastid/PM/vacuole (combined from (33, 53)) (Table II, Table S4). The YFP-GOT1 enrichment contained the highest proportion of previously identified proteins, likely due to the greater focus on Golgi-derived proteomes in these studies. To assist with comparisons between the sets of proteins associated with each of our baits and previously published proteomes we provide Table S4.

To identify possible cargo proteins from transient interactions of coat proteins and trafficking regulators, we extended our bioinformatic analysis to include predicted transmembrane domains and membrane associated modifications (Table S4). In the 433 proteins enriched by at least one of our affinity baits, we identified 64 proteins with one transmembrane domain and 58 with multiple transmembrane domains, 12 RAB GTPases with membrane association lipid modifications, and a diverse set of 63 potentially S-acylated proteins (52) (Table S4). The remaining 66% of our dataset (285 proteins) did not have any membrane association motifs and are putative cargo proteins. The proportions of proteins in each proteome with membrane association motifs varied across the enrichments; YFP-GOT1 had the greatest at 77%, while CLC2-GFP and YFP-RABG3f had the smallest proportion of membrane-associated proteins, 24 and 22%, respectively (Table II, Fig. S2). The proportion of YFP-GOT1 membrane-associated proteins is remarkably similar to Golgi or TGN proteomic studies such as Drakakaki et al. (44) (77%) or Parsons et al. (37) (75%). That our other affinity baits, such as RFP-RABF2b/ARA7, show lower proportions of membrane-associated proteins is likely to be a consequence of our choice of bait protein for affinity purification and the biological functions of these small GTPases. By targeting RAB regulators of vesicle trafficking, we identify more proteins with transient membrane interactions, such as vesicle coats (e.g. COPI and COPII), motor proteins (e.g. myosins), and endomembrane tethers than previous proteomic studies (Table III, Table S4).

**Affinity Enrichment of CLC2-GFP Identifies Clathrin-Mediated Endocytosis Components**—We included CLC2-GFP in this comparative proteomics study because we expected that the proteins associated with CLC2 would be substantially different to those enriched with RAB GTPase baits. Our CLC-GFP enrichment recovered established clathrin-interacting proteins and provides the first independent supportive evidence for the recently defined association of TPLATE complex components with clathrin (Table III). As expected, some of the most abundant proteins in the CLC2-GFP enrichments were; CHC1 (clathrin heavy chain 1, AT3G11130), CHC2 (AT3G08530), CLC1 (AT2G02760), and, the bait, CLC2 (AT2G40060). Several accessory proteins involved in CME were also assigned to the CLC2-GFP enrichment, including components from the TPLATE complex (47, 56) (Table III), specifically: two SH3 (SRC homology) domain-containing proteins (SH3PH, AT4G34660 and TASH3, AT2G07360), an epsin N terminal homology domain-containing protein ECA4 (AT2G25430), a WD40 protein (TWD-40–2, AT5G24710), and a calcium-binding EF hand protein EH2 (AT1G21630).

The TPLATE complex appears to persist at the plasma membrane after removal of AP2 and possibly through scis-
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Endomembrane regulators identified in affinity purifications. Proteins significantly enriched with each affinity purification are marked with a grey background and the fold enrichment over that of control baits is shown. Significant and fold enrichment was calculated using SAINT Express.

NS: proteins that were identified in an enrichment, but not significantly. A: No peptides to this protein were identified. Annotations for inclusion in a complex were manually curated from literature (26, 47, 65, 78, 79, 83, 101, 113–118)

| ATG number | Group | Short name | YFP-RABD2a/ARA5 | RABF1/ARA6-RFP | RFP-RABF2b/ARA7 | CLC2-GFP | YFP-GOT1 | YFP-RABG3f | YFP-VAMP711 |
|------------|-------|------------|-----------------|----------------|-----------------|----------|----------|------------|-----------|
| AT5G22780  | CCV   | Adaptin α2 | NS              | NS             | 11              | NS       | NS       | A          | A         |
| AT2G25430  | EGA4  | NS         | NS              | NS             | NS              | 73       | A        | 43         | NS        |
| AT4G32285  | CAP1  | NS         | NS              | NS             | 60              | NS       | A        | A          | A         |
| AT3G11130  | CHC1  | NS         | NS              | NS             | NS              | 29       | NS       | NS         | NS        |
| AT3G08530  | CHC2  | NS         | NS              | NS             | NS              | 60       | NS       | A          | A         |
| AT2G20760  | CLC1  | A          | NS              | NS             | NS              | 18       | A        | A          | A         |
| AT2G40060  | CLC2  | NS         | NS              | A              | 110             | NS       | A        | A          | A         |
| AT4G33650  | DPRA3 | 8          | NS              | 10             | 7               | NS       | NS       | NS         | NS        |
| AT1G10290  | DRA2A | NS         | NS              | NS             | NS              | NS       | NS       | 3          |           |
| AT4G34660  | SH3PH | A          | NS              | 40             | 40              | A        | A        | A          | A         |
| AT1G52360  | Coatamer | SEC27p  | 5               | 4              | 10              | NS       | NS       | 7          | NS        |
| AT4G31480  | SEC6   | NS         | A               | A              | NS              | A        | 11       | NS         |           |
| AT4G31490  | SEC6   | NS         | NS              | 12             | NS              | NS       | NS       | A          | A         |
| AT1G62020  | RET1p  | 16         | 16              | 21             | NS              | 12       | 7        |            |           |
| AT2G21390  | RET1p  | 6          | 7               | 9               | NS              | 6        | NS       |            |           |
| AT5G05010  | RET2p  | 10         | NS              | 10             | NS              | NS       | NS       |            |           |
| AT3G66340  | SEC31B | 8          | 6               | 10             | NS              | NS       | NS       | NS         |           |
| AT5G16300  | COG1   | COG1/VPS51 | 33              | NS             | 120             | NS       | NS       | NS         | NS        |
| AT4G24840  | COG2   | NS         | NS              | 37             | NS              | NS       | NS       | NS         | NS        |
| AT1G67930  | COG5   | NS         | NS              | 20             | NS              | A        | A        | A          | A         |
| AT5G51430  | COG7   | 27         | NS              | 50             | NS              | A        | NS       | NS         | NS        |
| AT5G11980  | COG8   | 30         | NS              | 47             | NS              | NS       | NS       | A          |           |
| AT2G27600  | ESCRT  | VPS4       | NS              | NS             | 7               | A        | NS       | 8          | A         |
| AT5G03540  | EXOCYST | EXO70A1 | NS              | NS             | 60              | NS       | A        | NS         | NS        |
| AT5G09730  | EXO70H7 | NS         | NS              | 30             | NS              | A        | A        | A          | A         |
| AT5G49830  | EXO84B/VPS51 | NS         | NS              | 40             | NS              | 23       | NS       |            |           |
| AT1G12370  | SEC10  | 9          | NS              | 12             | NS              | NS       | NS       | NS         | NS        |
| AT1G47550  | SEC3A  | NS         | NS              | NS             | NS              | NS       | 33       | NS         | NS        |
| AT1G76850  | SEC5A  | NS         | NS              | 107            | NS              | NS       | NS       | NS         | NS        |
| AT3G30380  | SEC8   | 20         | 12              | 29             | 10              | NS       | 12       | NS         |           |
| AT4G02030  | GARP   | VPS51      | NS              | NS             | 8               | NS       | A        | NS         | A         |
| AT1G71270  | VPS52  | 33         | 40              | 50             | 40              | NS       | 37       | NS         |           |
| AT1G50500  | VPS53  | 37         | NS              | 87             | NS              | 60       | NS       |            |           |
| AT4G19490  | VPS54  | 15         | NS              | 46             | NS              | NS       | NS       |            |           |
| AT3G60860  | GEF    | BIG2       | 24              | NS             | 60              | NS       | A        | NS         | NS        |
| AT1G01960  | BIG3   | 15         | NS              | 33             | 8               | NS       | 9        | NS         |           |
| AT3G43300  | BIG5/MIN7 | 16         | NS              | 23             | NS              | 11       | NS       |            |           |
| AT1G13980  | GNOM   | 53         | NS              | 97             | NS              | NS       | 33       | NS         |           |
| AT2G01470  | SEC12  | NS         | NS              | NS             | A               | NS       | NS       | 33         |           |
| AT1G16920  | GTPase | AtRABA1b   | 7               | NS             | 5               | NS       | NS       | NS         | NS        |
| AT4G18800  | AtRABA1d | NS        | NS              | 33             | NS              | NS       | 116      | NS         | NS        |
| AT3G46830  | AtRABA2c | 14         | NS              | NS             | A               | NS       | 38       | NS         | NS        |
| AT4G17170  | AtRABBC1c | NS        | NS              | NS             | NS              | NS       | 6        | 4          |           |
| AT1G43890  | AtRABCl | NS         | 12              | NS             | NS              | NS       | 6        | A          |           |
| AT3G11730  | AtRABD1 | A          | NS              | NS             | NS              | NS       | 30       | NS         |           |
| AT1G02130  | AtRABD2a | 56         | NS              | NS             | NS              | NS       | NS       | 7          |           |
| AT5G47200  | AtRABD2b | NS        | NS              | 10             | NS              | 11       | 7        |            |           |
| AT3G54840  | AtRABF1 | NS         | 195             | NS             | NS              | A        | NS       |            |           |
| AT4G19640  | AtRABF2b | 100        | 272             | 2330           | NS              | 76       | NS       |            |           |
| AT3G18820  | AtRABG3f | NS        | NS              | NS             | A               | 120      | 9        |            |           |
| AT2G44610  | AtRABH1b | NS        | NS              | NS             | NS              | NS       | 6        | NS         |           |
| AT3G56110  | RAB regulator | AtPRA1,B1 | NS             | A              | 117             | NS       | 53       | NS         | A         |
| AT2G40380  | AtPRA1,B2 | NS        | A               | NS             | 60              | NS       | A         |            |           |
| AT2G38360  | AtPRA1,B4 | NS        | NS             | 110             | A               | NS       | NS       | NS         | NS        |
| AT2G44100  | GDI1    | NS         | A               | 57             | A               | 657      | A         |            |           |
| AT3G59920  | GDI2    | NS         | A               | NS             | NS              | A        | 177      | A          |           |
We identified TPLATE components and dynamin (DRP3a, AT4G33650) in our CLC2-GFP enrichments, (but not AP2 or TPLATE itself), possibly capturing several different stages of interactions. Interestingly, we identified AP2, TPLATE, CAP1, and other members of this complex with RFP-RABF2b/ARA7 and YFP-RABD2a/ARA5 (Table III). Scission of vesicles from membranes is mediated by large GTPases such as dynamins, and we note that several dynamins were abundantly represented in many of our enrichments, including controls, and therefore did not pass our significance threshold. Only DRP3a (AT4G33650) passed significance thresholds with three baits, DRP3b (AT2G14120) with RFP-RABF2b/ARA7 and DRP2a (AT1G10290) with YFP-VAMP711.

One of the less-well-characterized proteins assigned to our CLC2-GFP enrichments was synaptotagmin A (SYTA, AT2G20990) that regulates endocytosis and plasmodesmal viral movement (57). Lewis et al. (57) demonstrated the colocalization of SYTA with the endocytic tracer dye FM4–64 and with RABF1/ARA6-GFP. We identified SYTA with CLC2-GFP, YFP-RABD2b/ARA5, and RFP-RABF2b/ARA7 baits. A. thaliana has five homologues of synaptotagmin, and little is known about the functions of this family. STY1 is the best characterized and functions in resealing.

**Fig. 3.** Sungear diagram of proteins assigned to the different proteomes. Sungear diagrams generated in virtual plant (http://virtual plant-prod.bio.nyu.edu/cgi-bin/sungear/index.cgi (51)). Groups of proteins are indicated by the black dots, with a size proportional to the number of proteins in the group. The arrows on each dot indicate the proteome assignment of a group of proteins. Enrichments were performed three times for each bait, proteins were accepted with SAINT scores >0.8.

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**TABLE III—continued**

| ATG number | Group | Short name | YFP-RABD2b/ARA5 | RABF1/ARA6-RFP | RFP-RABF2b/ARA7 | CLC2-GFP | YFP-GOT1 | YFP-RABG3f | YFP-VAMP711 |
|------------|-------|------------|-----------------|----------------|-----------------|----------|----------|------------|------------|
| AT3G19770  | VPS9a | A          | NS              | 130            | A               | A        | A        | A          | A          |
| AT4G30260  | YIP4b | 62         | NS              | 104            | A               | A        | A        | NS         | NS         |
| AT3G05280  | YIP5b | 80         | A               | NS             | A               | NS       | NS       | 60         | A          |
| AT1G75850  | RETROMER | VPS35A | NS              | A              | NS              | A        | A        | 40         | A          |
| AT2G17790  | VPS35B | NS         | NS              | NS             | A               | A        | 43        | A          | A          |
| AT5G06140  | SNX1  | NS         | NS              | 53             | NS              | A        | A        | NS         | NS         |
| AT2G45200  | SNARE | GOS12      | NS              | A              | A               | NS       | 40        | A          | A          |
| AT4G04910  | SYP43 | NS         | 70              | NS             | 113             | NS       | 70        | NS         | 93         |
| AT3G05710  | SYP51 | NS         | NS              | 23             | A               | A        | A        | NS         | 1713       |
| AT1G16240  | VAMP711 | A       | A               | A              | A               | A        | A        | 167        |            |
| AT3G32150  | VAMP721 | 33       | 9               | 16             | NS              | 6        | 18        | A          |            |
| AT3G54300  | VAMP727 | NS       | NS              | 40             | A               | A        | A        | A          |            |
| AT3G27590  | Tether | GC6       | NS              | 17             | NS              | NS       | NS       | A          |            |
| AT1G21630  | TPLATE complex | EH2 | NS              | NS             | 20             | A        | A        | A          |            |
| AT2G07360  | TASH3 | 8          | NS              | 6              | 5               | NS       | A        | A          |            |
| AT5G57460  | TML   | 23         | NS              | NS             | NS              | A        | A        |            |            |
| AT3G01780  | TPLATE | NS       | 7               | 10             | NS              | NS       | NS       |            |            |
| AT3G50590  | TWD40-1 | 6        | NS              | 8              | NS              | NS       | A         |            |            |
| AT5G24100  | TWD40-2 | NS       | NS              | 10             | A               | NS       | A          |            |            |
| AT5G11040  | TRAPP | TRS120     | NS              | 23             | NS              | A        | A        | NS         |            |
| AT5G16280  | TRS85 | 417        | NS              | NS             | NS              | A        | NS       | 53         |            |
| AT3G52850  | VSR   | NS         | NS              | 52             | A               | NS       | A         |            |            |
| AT2G14740  | VSR3  | A          | A               | A              | A               | A        | 47        |            |            |
| AT2G14720  | VSR4  | NS         | NS              | 147            | A               | A        | NS        |            |            |
| AT2G20990  | SYPA  | 48         | NS              | 26             | 46              | NS       | NS        |            |            |
| AT5G14950  | Golgi marker | GMII | NS              | NS             | 94              | NS       | A          |            |            |
| AT2G28520  | TGN marker | VHA-A1 | NS              | 50             | NS              | NS       | A          |            |            |

Fig. 3. Sungear diagram of proteins assigned to the different proteomes. Sungear diagrams generated in virtual plant (http://virtual plant-prod.bio.nyu.edu/cgi-bin/sungear/index.cgi (51)). Groups of proteins are indicated by the black dots, with a size proportional to the number of proteins in the group. The arrows on each dot indicate the proteome assignment of a group of proteins. Enrichments were performed three times for each bait, proteins were accepted with SAINT scores >0.8.
plasma membrane after stress (58, 59), and SYT2 is required for unconventional secretion, namely ER/Golgi-independent pathways and does not colocalize with FM4–64, RABF1/ARA6, or other endosomal markers but only with the Golgi apparatus (60).

The YFP-GOT1 Enrichment Is Consistent with Previously Published Golgi Proteomes—In all eukaryotes, the Golgi is an essential organelle for protein secretion, N-linked protein glycosylation and, in plants, is essential for the biogenesis and secretion of cell wall polysaccharides. YFP-GOT1 is a marker for the Golgi and is homolog of yeast GOT1p that localized to cis-Golgi (26, 61). Little is known about the precise location and function of GOT1 within the Golgi of A. thaliana. However, as several Golgi proteomic studies have been performed, we included YFP-GOT1 as an additional comparison for the RAB GTPase baits and expected it to be an outlier. We compared our YFP-GOT1 enrichment with published proteomic analyses of Golgi compartments (Fig. 4A, Table S4) (33, 37, 39, 53). Two thirds (41/62) of proteins identified with our YFP-GOT1 bait were previously identified in Golgi proteomic analyses (Fig. 4A).

In common with previously published Golgi proteomes, Golgi-localized enzymes were well represented in our YFP-GOT1 enrichment including: galacturonosyltransferases (GAUT, AT2G38650, AT3G02350, AT3G25140, AT3G61130), xylose synthase (AT2G47650) and xylene transferase (KKT5, AT1G74380), and a UDP-α-glucuronate 4-epimerase 6 (GAE1, AT5G47650) and xylose transferase (KKT5, AT1G26850, AT1G29790, AT4G00750, AT4G14360, AT5G64030, AT4G18030, AT1G26850, AT1G29470, AT3G23300). Nikolovski et al. (39) suggest that the SAM enzymes are involved in methylation of glycans. Four endomembrane family protein 70 (EMP70; AT4G12650, AT5G35160, AT5G25100, AT1G14670) were also identified in our YFP-GOT1 dataset. EMP70 proteins contain nine transmembrane domains, and their function is not known. Our YFP-GOT1 enrichment also contained the cis-Golgi-localized glycosyltransferase, alpha-mannosidase II (GMII, AT5G14950) that was not identified in previous Golgi proteomes. This protein is frequently used as a marker of the Golgi by Western blotting (e.g. (63)).

Although most of the proteins assigned to our YFP-GOT1 enrichment have been identified in published Golgi studies, our YFP-GOT1 enrichment contained fewer proteins (62 proteins) than were obtained by Parson et al. (37) (371 proteins), Nikolovski et al. (39) (197 proteins), or Sadowski et al. (53) (91 proteins). Our YFP-GOT1 enrichment shows an overlap of only seven proteins with the 146 proteins identified in the CFP-SYP61 TGN/EE proteome (44) and just four proteins with the TGN proteome (45) (Fig. 4A, Table S4). These differences suggest that YFP-GOT1 labels and enriches for a distinct area of the Golgi, possibly on the cis-Golgi side. To further test the specificity of our method, we compared the YFP-GOT1 enrichment to the YFP-VAMP711 enrichments (Fig. S3). These two proteomes should be distinct due to their spatial and functional differentiation to the Golgi and vacuole, respectively, and the data support this statement (as the H₀ of random assignment of proteins can be rejected with a X² - 43.3, p < .001).

As expected, the YFP-GOT1 enrichment differed from the proteins identified with our RAB baits and most obviously in that few of the known endomembrane regulatory proteins were identified with this bait (Table III). However, two RAB regulatory proteins (PRA1.B2, PRA1.B4) and a Qb-SNARE and a dissociation factor, (GOS12 and NSF) were identified with YFP-GOT1 and were also seen in previous studies. Additionally, the R-SNARE (VAMP722/SAR1) was identified with YFP-GOT1 (and four other of our baits) but was not identified in previous proteomic studies of the Golgi and TGN. Both of these SNARE proteins are known to localize to the Golgi, and in yeast, GOT1 facilitates SNARE fusion (64, 65) (Table III, Table S4).
Plants differ from animals in that they possess numerous highly mobile Golgi bodies that are tightly associated with the ER (66–69). COP-coated vesicles mediate traffic between the ER and the cis-Golgi and might be expected to be enriched with our GOT1 bait. However, the COP proteins we identified assigned to the small GTPase baits (RABD2a/ARA5 and RABF2b/ARA7). The structural roles of the proteins we selected as baits might explain this apparent discrepancy; GOT1 has four TM domains while the RAB GTPases associate transiently with membranes. In yeast, GOT1 is packaged into COPII vesicles and cycles between ER and Golgi (70). It is possible that the YFP-tag interfered with the GOT1 role in COPII vesicles or that the tag was masked from the affinity matrix by coatmers. In yeast, GOT1 has a role in membrane fusion, and we identified a novel fusigenic protein with YFP-GOT1, the large, dynamin-like, GTPase RH3 (AT3G13870). RH3 has homology to atlastins and is proposed to mediate ER tubule formation and fusion (60, 68, 71).

ARA5 and ARA7 RAB GTPase Mediator Trafficking Associated with the TGN—RAB GTPases cycle on and off membranes and recruit proteins (known as RAB effectors) such as coat proteins and tethers, to establish a unique, but transient, membrane identity. Our objective in selecting several RAB GTPases as baits for affinity purification was to capture some of the transient trafficking events associated with the TGN and not to characterize a steady-state composition, which has been established by Groen et al. (45). Of all our baits, the YFP-RABD2a/ARA5 and RFP-RABF2b/ARA7 enrichments showed the greatest similarity to the TGN proteomes identified using CFP-SYP61 and VHA-a1-GFP affinity purifications by (44, 45) (Fig. 4B, Table S4).

YFP-RABD2a/ARA5 is localized throughout the TGN and EE in punctate structures that are sensitive to BFA and is (partially) labeled with FM4–64 and colocalizes with VHA-a1 (26, 28). Of the 120 proteins we assigned to the YFP-RABD2a/ARA5 enrichments, 23 proteins were common to CFP-SYP61 and 17 to VHA-a1-GFP enrichments (Fig. 4B). We did not identify VHA-a1 in the YFP-RABD2a/ARA5 enrichment but did identify another VHA-A ATPase (AT1G78900); this protein was also identified in CFP-SYP61 affinity purifications (44). Surprisingly, there was substantial overlap between our YFP-RABD2a/ARA5 and RFP-RABF2b/ARA7 enrichments (85 proteins). The RFP-RABF2b/ARA7 enrichment did not contain RABD2a but did contain RABD2b possibly supporting separate roles for these two RABD2 proteins as reported by (28).

Our RFP-RABF2b/ARA7 enrichments showed an overlap of 20/105 proteins that were identified in VHA-a1-GFP affinity enrichments by Groen et al. (45), including VHA-a1 (AT2G28520). VHA-a-1 was also identified in CFP-SYP61 enrichments by Drakakaki et al. (44), and our RFP-RABF2b/ARA7 enrichment had an overlap of 35/146 with the CFP-SYP61 proteome (44). That we did not identify SYP61 in any of our seven affinity enrichments could suggest distinctness of the proteomes associated with each bait. The 279 RFP-RABF2b/ARA7-associated proteins were the most numerous and diverse of any of our enrichments, suggesting that RABF2b/ARA7 might have a general role in EE/TGN and LE/MVB trafficking.

RAB GTPase Regulator Proteins Exhibit Varied Associations—We identified many proteins that regulate RAB GTPase function in YFP-RABD2a/ARA5 and RFP-RABF2b/ARA7 enrichments (Table III). All RAB GTPases have a hydrophobic membrane association modification, typically prenylation, and as membrane association is a prerequisite for RAB GTPase activation by exchange of GDP for GTP, proteins that bind to this motif prevent the RAB from associating with the membrane. Therefore, these regulatory proteins are known as GDP dissociation inhibitors (GDIs; reviewed in (72–74)). We identified GD1 with RFP-RABF2b/ARA7 enrichments. For a RAB GTPase to associate with a membrane, the GDI must be removed by the action of a GDI dissociation factor (GDF) (70, 75–77). The homologs of yeast GDFs in A. thaliana are the Ypt-interacting protein (YIP) and prenylated Rab GTPase acceptor 1 (PRA1) family (78, 79). We identified YIP4b with both RFP-RABF2b/ARA7 and YFP-RABD2a/ARA5 baits and PRA1.B4 and PRA1.B1 with RFP-RABF2b/ARA7 and YIP5b and YFP-RABD2a/ARA5. GD1 was not identified in either CFP-SYP61 or VHA-a1-GFP proteomes, as expected for a protein that associated with the cytoplasmic complex of a RAB. The PRA1 and YIP proteins were identified in previously characterized TGN proteomes.

RABs are poor GTPases, and their activity at membrane surfaces is controlled by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) that facilitate nucleotide exchange and hydrolysis. We identified VPS9a, the activating GEF for RABF GTPases (80) (and YFP-RABG3f) enrichments. The interaction of RABF2a/ARA7 with VPS9a is well characterized: In yeast-two hybrid assays and by coimmunoprecipitation, VPS9a physically interacts with RABF proteins (80) and the structure of the RABF2b/ARA7–VPS9a complex has been solved (81). In the YFP-RABD2a/ARA5 enriched proteins, we identified one core Transport Protein Particle subunit I (TRAPPI) component, TRSS85 (AT5G16280) (82). There are two distinct TRAPP complexes in animal cells: TRAPPI and TRAPPII, and in yeast, the TRAPPI complex is GEF to the RAB1/RABD homologue, Ypt1p (83, 84). Due to their coenrichment, we suggest that the TRAPPI may also be a GEF for RABD2a/ARA5. TRAPP components were identified in CFP-SYP61 and VHA-a1-GFP proteomes, but VPS9a was not.

We identified four other GEFs, of the ADP-ribosylation factor (ARF) family, in both YFP-RABD1a/ARA5 and RFP-RABF2b/ARA7 enrichments, specifically GNOM (AT1G13980), BIG5/Min7 (AT3G43300), Big2 (AT3G60860), and Big3 (AT1G01960). These four GEFs can be all be inhibited by BFA and are associated with coated vesicle formation (1). The best-known plant ARF-GEF is GNOM due to its regulation of auxin transporter (PIN) recycling and partial colocalization with FM4–64.
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and (85). GNOM has recently been shown to be primarily localized to the Golgi and to regulate TGN structure (86). BFA treatment seems to pull the Golgi into a body that is surrounded by TGN, and this is consistent with recent work disambiguating TGN structure (87) and suggests that GNOM influence on ARA7 localization is indirect (85, 86). Less is known about the functions of BIG1 to BIG4, and BIG5/MIN7 has a role in plant immunity and PIN trafficking at the TGN/EE (88, 89). Of these four ARF-GEFs, only BIG5/MIN7 was previously identified in the VHA-a1-GFP affinity purification.

Identification of Candidate Novel GARP Components—RAB GT-Pases regulate compartment identity by recruiting intramembrane tethers and SNARE proteins, which promote the direct fusion of membranes once tethering factors have brought them into close contact, reviewed in (87). The combinations of RAB, tethers, and SNARE proteins dictate the interactions a compartment can make and are further regulated by SNARE attachment proteins (SNAP) and Sec1/Munc18 (SM) proteins.

We identified tether proteins and complexes with RFP-RABF2b/ARA7 and YFP-RABD2a/ARA5 baits, including: conserved oligomeric Golgi (COG), Golgi-associated retrograde protein (GARP), and the golgin GC6 (with RFP-RABF2b/ARA7 only). The COG and GARP are both multisubunit complexes of the Complex Associated with Tethering Containing Helical Rods (CATCHR) group of long tethers and are known to be effectors of RAB GT-Pases (90). These complexes were not identified in previous proteomic analyses of the TGN.

In yeast, the GARP complex coordinates retrograde Golgi traffic and interacts with RAB, SNARE, and golgin proteins (reviewed in (88, 91, 92)). In plants, homologues of the COG complex have been predicted on the basis of sequence similarity (93), but there is little experimental evidence for the function and location of this complex in A. thaliana. We identified five of the eight putative COG subunits with RFP-RABF2b/ARA7 (COG1, COG2, COG5, COG7, COG8) and two with YFP-RABD2a/ARA5 enrichments (COG 7, COG8; Table III). The A. thaliana COG2 (AT4G24840) subunit has recently been shown to recruit the exocyst subunit EXO70A1 to punctate structures at cortical microtubules during xylem formation (94). The putative COG1 subunit (AT5G16300) has also been annotated as a possible GARP complex subunit (VPS51) (95). We have placed AT5G16300 in the COG group of Table III, but it could equally belong to the GARP complex.

The primary role of the tetrameric GARP complex is in the reception of endosome-derived vesicles at the TGN (recently reviewed in (90)). GARP is a highly conserved complex consisting of VPS51, VPS52, VPS53, VPS54 subunits (96)). In yeast, the VPS52 subunit interacts with GTP bound RAB, Ypt6 (97, 98); RABH is the homologous group in A. thaliana. We identify the VPS52 subunit with five of our affinity enrichments (RABF1/ARA6-RFP, YFP-RABG3f and CLC2-GFP baits in addition to YFP-RABD2a/ARA5 and RFP-RABF2b/ARA7), whereas the other three GARP components appear to have a more limited distribution (Table III). We suggest that VPS52 might be the GT-Pase-interacting component of the GARP complex in plants. VPS51 (Unhinged; At4g02030) has recently been shown to interact directly with VPS52 and to colocalize with RABF2a and SYP61 (95). A. thaliana contains three putative VPS51 homologues, and the identification of VPS51 with the other three GARP subunits in our RFP-RABF2b/ARA7 enrichments further supports Pahari et al. Notably VPS51 is not significantly enriched with YFP-RABD2a/ARA5, but another putative VPS51/COG1 homologue (AT5G16300) is.

The Transition from the TGN/EE to LE/MVB—To the best of our knowledge, there are no comparable published proteomes to our YFP-RABG3f, YFP-VAMP711, and RABF1/ARA6-RFP enrichments. Analysis of these proteomes should help elucidate the biological mechanisms of transition from RAB5-labeled LE/MVBs to RAB7-labeled LE/MVBs. Furthermore, 167 proteins of our RFP-RABF2b/ARA7 enrichments did not overlap with TGN-associated proteomes (Fig. 4B). These four baits have many proteins in common and represent the late endosomes to the LE/MVB and vacuole (Fig. 5B).

One striking observation is that the proteomes of RFP-RABF2b/ARA7 and RABF1/ARA6-RFP are very, but not significantly (X² - 0.6, p > .1), similar with an overlap of 49 proteins (Fig. 5A). The RABF1/ARA6-RFP proteome is much smaller than RFP-RABF2b/ARA7, with just 14 proteins not seen in RFP-RABF2b/ARA7, and only eight proteins are unique to RABF1/ARA6-RFP (compared with all our baits).
These eight proteins are; TRS120, two ARM repeat family proteins (AT1G65220, AT3G05040), two chloroplast-associated proteins (LHC, AT3G08940; TOC, AT3G46740), and three enzymes (PFK, AT4G29220; KCS19, AT5G04530; GPTA4, AT1G01610). The RABF regulator VPS9a (AT3G19770) was identified with both baits, but only passed our significance test with RFP-RABF2b/ARA7 (80). An important difference between the RFP-RABF2b/ARA7 and RABF1/ARA6-RFP endosomes is the presence of numerous TGN/EE-localized proteins (RABA1b, SYP43, VHA-a1, SCAMP1) and many long tether complexes in the RFP-RABF2b/ARA7 but not RABF1/ARA6-RFP enrichments (8, 72, 99).

Fig. 6. Co-localizations of PRA1 family members and RABF1/ARA6 (LE/MVB), RABF2b/ARA7 (LE/MVB) and GOT1 (Golgi). Standard confocal micrographs of leaf epidermis of the indicated A. thaliana transgenic plants stably expressing pUB::YFP-GOT1, RFP-RABF2b/ARA7, or RABF1/ARA6-RFP, transiently transformed using particle bombardment, expressing fluorescent tagged PRA1 family members. Insets show an enlarged section of each image.
That the proteins identified with RABF1/ARA6-RFP are nearly a subset of RFP-RABF2b/ARA7 is in accordance with observations that RABF2b endosome populations mature into the RABF1/ARA6 endosomes (25, 29, 46, 100). Despite the similarities in their associated proteins, RABF1/ARA6- and RABF2b/ARA7-labeled LE/MVBs are distinct. RABF2b/ARA7 LE/MVBs are more susceptible to application of the drug BFA than RABF1/ARA6 LE/MVBs (25). Furthermore, RABF2b/ARA7 colocalizes to a greater extent with the SNARE VAMP727 while RABF1/ARA6 colocalizes to a greater extent with the SNAREs SYP21 and SYP22 (25). The BFA-sensitive ARF-GEFs and VAMP727 were significantly enriched with RFP-RABF2b/ARA7 but not with RABF1/ARA6-RFP. An alternative explanation for some of the differences between our RFP-RABFb/ARA7 and RABF1/ARA6-RFP enrichments might be due to the expression level of these constructs or in their relative efficiency in binding to the affinity matrix.

The YFP-RABG3F enrichment contained 136 proteins and has an overlap of 78 proteins with RFP-RABF2b/ARA7-identified proteins and the RABF1/ARA6-RFP and YFP-VAMP711 baits share 34 and 25 proteins, respectively, with this enrichment (Fig. 5B). The greatest diversity of RAB proteins was identified with RABG3F enrichments, but only two of the five ARF GEFs were identified in total (Table III and Table S4). Other RAB proteins identified with YFP-RABG3f included members of the RABA, RABC, RABD, RABF, and RABH families. The RAB dissociation inhibitors GDI1 and GDI2 were abundantly represented with the YFP-RABG3f bait (assessed by fold enrichment over control samples), and YFP-RABG3f was the only bait to enrich for GDI2. Forty-one other proteins were specific to the YFP-RABG3f proteome (compared with all seven baits).

The LE/MVB is characterized by luminal vesicles that are formed by the endosomal sorting complex required for transport (ESCRT) complex. We did not identify any core ESCRT components with YFP-RABG3f enrichments, but the ATPase that promotes the disassembly of the ESCRT complex, VPS4, was identified with RFP-RABF2b/ARA7 and YFP-RABG3f baits. Two components of the retromer complex were identified only with the YFP-RABG3f enrichments (VPS35A, VPS35B). The retromer recycles vacuolar sorting receptors back to the Golgi/TGN. Zelazny et al. demonstrated that VPS35 interacts with RABG3f. The retromer consists of two subcomplexes; VPS35-VPS26-VPS29 and a sortin nexin (SNX) (reviewed (101)). We did not identify VPS26 or VPS29 in any enrichment, but we did identify SNX1 with RFP-RABF2b/ARA7, which is known to colocalize with them (102). In addition, two cargo recognition vacuolar sorting receptors, (VSR1 and VSR4) were identified with RFP-RABF2b/ARA7 enrichment only, and VSR3 was identified with YFP-VAMP711 baits. These VSRs are involved with sorting cargo from the TGN to the lytic vacuole and to the seed protein storage vacuole (103).

SNAREs at the Vacuole—Our YFP-VAMP711 enrichment contains 51 proteins, of which 12 are unique to VAMP711. Interestingly, we identified only two SNARE proteins in the YFP-VAMP711 enrichment, VAMP721 and SYP51. These three proteins cannot form a partial SNARE complex, as both VAMP711 and VAMP721 are both R-SNAREs (72). SYP51 is, however, a known interaction partner of VAMP711 and may function as an “interfering” SNARE (104). Of particular interest is the identification of SEC12 in the YFP-VAMP711 proteome; this GEF has been implicated in regulating the exit of vacuolar proteins from the ER. It may be that the vacuole represents another localization for SEC12 (105).

Microscopic Analysis of Golgi and TGN/EE Copurifying Proteins—To verify that copurifying proteins are present at the compartments affinity purified with our baits, we monitored the subcellular localization of some of the identified proteins using confocal microscopy. We focused on the YFP-GOT1 and RFP-RABF2b/ARA7 proteomes and selected PRA1 proteins for further study as they are believed to regulate RAB GTPase function through removing the inhibitory GDI (78), although direct evidence is lacking in plants (106). In A. thaliana, the PRA1 family has expanded to include 19 members and their subcellular localizations are thought be varied (78, 107). We identified PRA1.B1 (AT3G56110) in both the YFP-GOT1 Golgi and RFP-RABF2b/ARA7 proteomes, whereas PRA1.B2 (AT2G40380) was found in the YFP-GOT1 proteome only (Table III). By contrast, no spectrum counts matching peptides for PRA1.F1 (AT1G17700) were identified in any of our proteomes (Table S4), although it belongs to the same protein family as PRA1.B1 and PRA1.B2 (78). These observations suggest distinct localization patterns among the PRA1 proteins of the same family.

Using live-cell imaging, we observed partial colocalization of YFP-/RFP-tagged PRA1.B1 with both RFP-RABF2b/ARA7 and YFP-GOT1, respectively (Fig. 6, Fig. S4). Additionally, YFP-RFP-PRA1.B1 was present at some compartments that were not positive for YFP-GOT1 or RFP-RABF2b/ARA7, showing PRA1.B1 has a wider subcellular localization to both Golgi and endosomes. RFP-PRA1.B2 was predominantly colocalized with YFP-GOT1 (Fig. 6, Fig. S4), in agreement with predictions from our proteomic analysis. YFP/RFP-PRA1.F1 was found to be closely associated with YFP-GOT1, RFP-RABF2b/ARA7, and RABF1/ARA6-RFP-positive compartments while not exhibiting strong colocalization (Fig. 6, Fig. S4), which is consistent that this PRA1 protein was not identified in our endomembrane proteomes. All these observations corroborate the findings from our proteome analysis and demonstrate that our data can be used to predict the localization of a protein within the cell. In contradiction to our results that RABF2b/ARA7 and PRA1.F1 do not colocalize, a previous study reports colocalization between mRFP-ARA7 and PRA1.F1-GFP. This is likely to be due to the differences in the systems used for expression (78).
Recent reviews and discussions (13, 54, 108) have highlighted the need to identify additional regulatory proteins and cargo of endosomal and secretory pathways. To meet this need, Table III and Table S4 provide a potential resource for the community to explore the proteomes we present here in the context of published datasets. Our results demonstrate the utility of affinity purification for the exploration of endomembrane proteomes, and our method is comparable in efficacy to other endomembrane enrichment protocols. Furthermore, our proteomic data yield unprecedented biologically significant resolution between separate endocytic compartments (for example, the common and specific proteins in Fig. 5B) and can be used to predict the localization of proteins as evidenced by the PRA1 family localizations (Fig. 6).

It is important to note that endosomes and membrane fractions associated with a specific marker protein exist as heterogeneous populations within the cell and that affinity purification will pool these populations. For example, we identify GDI1 and GDI2 that only associate with the nonmembrane fraction of prenylated RABs, and we identify PRA1 family proteins with YFP-RABG3f and RFP-RABF2b/ARA7. PRA1 proteins are GDFs involved in the positive regulation of RAB GTPases by removing the inhibitory GDIs. We demonstrate differential localization of three related PRA1 GDFs and find there were no recognizable GDFs in the RABF1/ARA6-RFP proteome and that neither PRA1.B1, B2 or F1 colocalize with ARA6 (Fig. 6) when analyzed with confocal microscopy. As ARA6 has a different lipid association modification (n-myristolation) (29) to all other RABs (prenylation) (72) and the known GDF machinery is believed to regulate RABs through interaction with the prenylation modification (78), we hypothesize that ARA6 has a set of as yet unidentified noncanonical GDF machinery. Furthermore, we identify known cargoes of endosomes such as PIN3 (109), PEN3 (110), and CESA (111) (Table S4). We emphasize that our data are a starting point for further work and not an end point in itself; additional validation from the community is required to properly establish the proteomes we have pioneered.

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[This article contains supplemental material Tables S1 to S4 and Figs. S1 to S4.]

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