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AtVPS45 Complex Formation at the trans-Golgi Network

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The Sec1p family of proteins are thought to be involved in the regulation of vesicle fusion reactions through interaction with t-SNAREs (target soluble N-ethylmaleimide–sensitive factor attachment protein receptors) at the target membrane. AtVPS45 is a member of this family from Arabidopsis thaliana that we now demonstrate to be present on the trans-Golgi network (TGN), where it colocalizes with the vacuolar cargo receptor AtELP. Unlike yeast Vps45p, AtVPS45 does not interact with, or colocalize with, the prevacuolar t-SNARE AtPEP12. Instead, AtVPS45 interacts with two t-SNAREs, AtTLG2a and AtTLG2b, that show similarity to the yeast t-SNARE Tlg2p. AtTLG2a and -b each colocalize with AtVPS45 at the TGN; however, AtTLG2a is in a different region of the TGN than AtTLG2b by immunogold electron microscopy. Therefore, we propose that complexes containing AtVPS45 and either AtTLG2a or -b define functional subdomains of the TGN and may be required for different trafficking events. Among other Arabidopsis SNAREs, AtVPS45 antibodies preferentially coprecipitate AtVTI1b over the closely related isoform AtVTI1a, implying that AtVTI1a and AtVTI1b also have distinct functions within the cell. These data point to a functional complexity within the plant secretory pathway, where proteins encoded by gene families have specialized functions, rather than functional redundancy.

INTRODUCTION

The plant secretory pathway consists of a series of membrane-bound organelles through which protein and membrane traffic flows via small transport vesicles (for review, see Sanderfoot and Raikhel, 1999). Most soluble cargo proteins enter the system at the endoplasmic reticulum and are transported through the Golgi apparatus to the trans-Golgi network (TGN). Vesicles budding from the TGN have various destinations. Secretion is thought to be the default pathway, and secreted proteins are transported in vesicles that fuse with the plasma membrane and release their contents to the outside of the cell. Membrane components may then be retrieved from the plasma membrane via the endocytic pathway. Clathrin-coated vesicles containing some vacuolar proteins also bud from the TGN and transport their cargo to the prevacuolar compartment (PVC). In this case, a sorting signal within the cargo protein is required, which is usually at the N terminus of the protein. The signal is recognized by a cargo receptor protein in the TGN, which directs its packaging into clathrin-coated vesicles for transport to the PVC. By analogy with yeast and mammalian systems, after fusion of the vesicle with the PVC, the cargo is thought to be released from the receptor and transported on to the vacuole, whereas the receptor is recycled back to the TGN, probably via another vesicle transport and fusion reaction (Bryant and Stevens, 1998; Sanderfoot and Raikhel, 1999). Another level of complexity is added in some plant cells, which may contain two or three different types of vacuoles. This was observed morphologically a number of years ago, and more recently it has been substantiated by the use of antibodies specific to each vacuole type (for review, see Vitale and Raikhel, 1999). Additional transport pathways carry vacuolar storage proteins, which may contain a C-terminal or internal vacuolar sorting signal, in smooth dense vesicles from the endoplasmic reticulum or Golgi apparatus to the storage vacuoles. Even in cells that appear to contain only a single large central vacuole, at least two mechanisms exist for the transport of soluble proteins to that vacuole. Proteins containing a C-terminal vacuolar sorting signal are transported via a wortmannin-sensitive mechanism, whereas the transport of proteins containing an N-terminal signal is insensitive to wortmannin (Matsuoka et al., 1995). A third pathway is thought to transport vacuolar membrane

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Abbreviations used: Cvt, cytoplasm-to-vacuole trafficking; HA, hemagglutinin; PVC, prevacuolar compartment; SNARE, soluble N-ethylmaleimide–sensitive factor attachment protein receptor; TGN, trans-Golgi network.

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D.C. Bassham et al.

proteins from the TGN (Gomez and Chrispeels, 1993). Many transport pathways thus operate in a single plant cell at the same time, involving many different vesicle and organelle types. Central questions remain regarding how the fidelity of vesicle transport is maintained and how a vesicle is able to fuse with its target membrane, and only its target membrane, out of the many organelle types in the cell.

In the study of vesicle fusion in different organisms and different transport steps, some central themes have emerged (Rothman, 1996). Families of conserved membrane proteins have been identified on the vesicles (v-SNAREs [soluble N-ethylmaleimide–sensitive factor attachment protein receptors]) and target organelles (t-SNAREs) that interact upon docking of the vesicle at the target membrane and are essential for fusion. In fact, in a minimal in vitro system, v- and t-SNAREs alone were found to be sufficient for membrane fusion (Weber et al., 1998; Nickel et al., 1999; Parlati et al., 1999). Different (although related) v- and t-SNARE functions in most of the transport steps within a cell. In vivo, two soluble proteins, NSF (N-ethylmaleimide–sensitive factor) and SNAP (soluble NSF attachment protein), are also required for fusion and function in most transport steps in the cell. Although these membrane and soluble proteins appear to make up the core fusion machinery, many other proteins are required in vivo for vesicle transport and may regulate the fusion of a vesicle with its target membrane. One family of proteins that may fulfill this role is the Sec1p family. The gene encoding Sec1p, the original member of this family, was identified in a screen for yeast mutants defective in secretion (Novick and Schekman, 1979). Sec1p is a hydrophilic protein that is required for the fusion of secretory vesicles with the plasma membrane and interacts with the plasma membrane t-SNAREs Sso1p and Sso2p (Aalto et al., 1993). Recent evidence suggests that yeast Sec1p can associate with the intact SNARE complex containing both t- and v-SNAREs (Carr et al., 1999). However, studies in mammalian cells demonstrate that the v-SNARE is excluded from the syntaxin/nSec1 complex (Pevsner et al., 1994a,b; Yang et al., 2000), and the precise function of Sec1p and Sec1p-like proteins is still unclear.

In addition to Sec1p itself, three other Sec1p-like proteins are found in yeast and function in a variety of transport steps. They are all believed to act in vesicle fusion and to interact with the t-SNARE at the target membrane. Vps45p is a Sec1-like protein involved in vesicle transport to the yeast vacuole. In a vps45 mutant, the normally vacuolar hydrolase carboxypeptidase Y is secreted and small transport vesicles accumulate (Cowles et al., 1994; Piper et al., 1994). Consistent with a role in transport from the TGN to the vacuole, Vps45p interacts with the prevacuolar t-SNARE Pep12p and the FYVE domain–containing protein Vac1p, both of which are involved in this transport step (Burd et al., 1997; Peterson et al., 1999; Tall et al., 1999). However, Vps45p also associates with the TGN or endosomal t-SNARE Tlg2p, and Tlg2p appears to be the principal binding site for Vps45p on membranes (Nichols et al., 1998). The Tlg2p-Vps45p complex is required for cytoplasm-to-vacuole trafficking (Cvt) of aminopeptidase I at an early stage of the pathway and has been proposed to be involved in the formation of Cvt transport intermediates (Abeliovich et al., 1999). Tlg2p and Vps45p are also required for endocytic recycling of the v-SNARE Snclp to the plasma membrane (Lewis et al., 2000). Vps45p, therefore, appears to have more than one function in the late secretory pathway in yeast.

We have previously isolated a cDNA (AtVPS45) encoding a Vps45p-like protein from Arabidopsis thaliana that is able to complement the vacuolar sorting defect of the yeast vps45 mutant (Bassham and Raikhel, 1998). On sucrose density gradients, AtVPS45 cofractionates with the vacuolar cargo receptor AtELP (Ahmed et al., 1997), which probably cycles between the TGN and the PVC (Sanderfoot et al., 1998), but not with the prevacuolar t-SNARE AtPEP12 (Conceição et al., 1997; Bassham and Raikhel, 1999), indicating that AtVPS45 and AtPEP12 may reside on distinct membrane populations. We now demonstrate that AtVPS45 is found on the TGN in Arabidopsis roots, where it colocalizes with AtELP by immunogold electron microscopy. AtVPS45 interacts with two newly identified Tlg2p-like proteins from Arabidopsis, AtTLG2a and AtTLG2b, and not with AtPEP12. Consistent with this localization, AtTLG2a and AtTLG2b also reside on the TGN, although they appear to be present in different subdomains of the organelle. Both AtTLG2a and AtTLG2b partially colocalize with AtVPS45. One other SNARE, AtVTI1b, also communoprecipitates with AtVPS45 antibodies and thus appears to be present in the AtTLG2a (or AtTLG2b)-AtVPS45 complexes. Therefore, we propose that AtVPS45 and AtVTI1b function together in a vesicle fusion reaction with AtTLG2a or AtTLG2b at the TGN in Arabidopsis.

MATERIALS AND METHODS

Electron Microscopy Procedures

Cryosections of Arabidopsis root tips were prepared as described by Sanderfoot et al. (1998) and used for all immunogold labeling experiments. Immunolabeling was performed as described by Sanderfoot et al. (1998) and Zheng et al. (1999b). For double-labeling experiments, after incubation of the grids with the first antibody, a second fixation step followed by a second blocking step was used to prevent cross-reactivity of the antibodies at later stages of the protocol. For each combination of antibodies, controls were used with the corresponding preimmune serum substituted for one or both of the antisera. In all cases, these controls demonstrated that the labeling seen was highly specific.

Isolation and Cloning of Three Novel Arabidopsis t-SNAREs

Analysis of the amino acid sequences of many syntaxin-type t-SNAREs from yeast, mammals, and plants has shown that the coiled-coil region near the C-terminal transmembrane anchor is highly conserved. A consensus protein sequence derived from this region was used to search sequence databases (tBLASTn, www.ncbi.nlm.nih.gov) for new Arabidopsis sequences that may represent t-SNAREs. With this consensus sequence, all of the previously characterized Arabidopsis t-SNAREs (AtPEP12 [Bassham et al., 1995], AtVAMS [Sato et al., 1997], AHPPL [Zheng et al., 1999a], KNOLLE [Lukowitz et al., 1996], and AtSYRI [Leyman et al., 1999]) were found as cloned cDNAs, expressed sequence tags, or genomic sequences. Other sequences were found that either represented additional homologues of the known t-SNAREs or that represented novel Arabidopsis t-SNAREs.

Two of these novel sequences, corresponding to the predicted genes F2P16.16 and T10 M13.19 (found on bacterial artificial chromosomes from chromosomes V and IV, respectively), were found to be highly homologous to each other and were each most related to the yeast t-SNARE ScTlg2p and to mammalian Syntaxin 16.
these yeast and mammalian t-SNAREs are localized to late Golgi compartments, it was likely that these Arabidopsis t-SNAREs would also be found on a late Golgi compartment; therefore, they were investigated further. Because of this homology, we related to the genes encoding these t-SNAREs as AtTLG2a (F2P16.16) and AtTLG2b (T10 M13.19). AtTLG2a was found to be encoded by an expressed sequence tag that was acquired from the Ohio State Stock Center (Columbus, OH). AtTLG2b was not represented by an expressed sequence tag; thus, to isolate a cDNA, primers were designed to sequences 5′ and 3′ of the ORF (TLG2a-F1: GCC CTC GAG CAG AGG GTC ATT TGC ATT TTC CT; TLG2b-R1: GCC CAA GAG AGG GTC ATT GTC TGT TAC) and used to amplify a product from total RNA extracted from Arabidopsis roots by reverse transcriptase–PCR according to the manufacturer’s protocol (Life Technologies, Grand Island, NY). This product was cloned into pGEM-TEasy (Promega, Madison, WI) according to the manufacturer’s protocol.

To aid in further studies with AtTLG2a and AtTLG2b, the cDNAs of each were modified by PCR to insert restriction sites at the 5′ and 3′ ends of the ORFs. Specific primers were used to place XhoI and NdeI restriction sites directly upstream of the start codon and an Xhol site immediately after the stop codon (TLG2a-F: GG TCT AGA CAT ATG GCG ACG CAT ATG GCG ACG AGG AA; TLG2b-F2: GG TCT AGA CAT ATG GCG ACG CAT ATG GCG ACG AGG AA; TLG2b-R2: GG CTC GAG GAC TAA AAC ATA TTC CT; TLG2a-R: GG CTC GAG TCA CAA GAG CTA AGC CAC AAA GAA GAA GG). This amplified product was cloned into pBluescript KS (Stratagene, La Jolla, CA), creating pNde-TLG2a and pNde-TLG2b.

A third novel sequence, corresponding to a predicted gene (Mj1596) from the M. jannaschii chromosome V phage artificial chromosome, encoded a protein that showed high homology to t-SNAREs known to be localized to the cis-Golgi (yeast Sed5p and mammalian Syntaxin 5; see Sanderfoot et al., 1999) and was named AtSED5. A cDNA corresponding to this gene was isolated by reverse transcriptase–PCR with the use of specific primers that inserted HindIII and NdeI sites at the 5′ end and an Xhol site at the 3′ end of the ORF (Seds-F: GG AAC CTT CAT ATG CCC AGG AGG AA; Seds-R: CC CTC GAG GAC TAA AAA AAA ATA TTC). The amplified products of these reactions were separately cloned into pBluescript KS (Stratagene, La Jolla, CA), creating pNde-TLG2a and pNde-TLG2b.

For expression of Arabidopsis t-SNAREs

Theplasmid pH6-Nde is a derivative of pBluescript KS in which the sequence between the BamHI and PsI restriction sites has been replaced by a linker encoding a start codon followed by seven histidine residues and an NdeI restriction site (GATCA ATG CAT CAT CAT CAT CAT CAT ATG CTGCA). The NdeI–Xhol fragments of pNde-TLG2a, pNde-TLG2b, and pNde-SED5 were separately cloned into similarly digested pH6-Nde to create pH6-AtTLG2a, pH6-AtTLG2b, and pH6-SED5. The NdeI–HindIII fragment of pH6-PepP2 (Sanderfoot et al., 1999) was cloned into similarly digested pH6-Nde to create pH6-AtPEP12. The BamHI–EcoRI fragments of a plasmid containing the AtVAM3 cDNA, which was engineered to contain a BamHI restriction site immediately upstream of the start codon (Sanderfoot et al., 1999), was cloned into pET-28a, creating pH6-AVAM3. The DNA encoding these 6xHis-tagged t-SNAREs was subsequently cloned into pVT102-U (Vernet et al., 1987) and introduced into yeast as described previously (Sanderfoot et al., 1999). Expression of each of these t-SNAREs was confirmed with the use of mAbs specific to sequences 5′ and 3′ of the ORF (TLG2a-R1: GGC CAA GAG AGG GTC ATT GTC TGT TAC) and with specific antisera to each t-SNARE, where available.

For expression of untagged AtTLG2a and AtTLG2b in yeast cells, the Xhol–Xhol fragments of pNde-TLG2a and pNde-TLG2b were separately cloned into pVT102-U and introduced into yeast.

### Binding of AtVPS45p to Arabidopsis t-SNAREs Expressed in Yeast

The complete ORF of AtVPS45 was subcloned into the yeast expression vector PG-1 (Schenk and Yamamoto, 1988) and introduced into yeast strains containing the His-tagged t-SNAREs (see above) or pVT102-U vector as a control. Each double transformant was analyzed for expression of AtVPS45 with the use of specific antibodies and for expression of the tagged t-SNARE with the use of 6xHis mAbs.

Cells from 10-ml overnight cultures of each of the transformants were resuspended in 1 ml of lyticase solution (0.1 mg/ml lyticase [Sigma Chemical, St. Louis, MO], 100 mM KPO4, pH 7.5, 1.2 M sorbitol) and digested for 2 h at 37°C. Spheroplasts were lysed by vortexing with glass beads in binding buffer (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 5 mM imidazole, 1% [vol/vol] Triton X-100) followed by a 2-h incubation at 4°C to solubilize membrane proteins. Debris was pelleted by centrifugation for 5 min at 13,000 × g, and the supernatant was rocked for 3 h with a 25-μl bed volume of His-bind resin (Novagen, Madison, WI) to allow the His-tagged t-SNAREs to bind to the resin. The beads were washed five times in 1 ml of binding buffer, followed by elution of bound protein with binding buffer containing 200 mM imidazole. The eluates were precipitated with trichloroacetic acid and analyzed by SDS-PAGE and immunoblotting. Coprecipitation of AtVPS45 with the t-SNAREs was detected with the use of specific AtVPS45 antibodies.

### Production of Antiserum against AtTLG2a

To produce protein in bacteria for use in generating antibodies, further modification of the AtTLG2a cDNA was performed. TGL2a-F was used in combination with a primer that inserted an Xhol restriction site immediately before the first codon of the transmembrane anchor (TGL2a-R: TCA CTG GAG GTC ATT CAT ACC TCA CCC ACC TAA AAA AAA ATA TTC). This amplified product (which encodes for amino acids 1–299 of AtTLG2a) was cloned into Xhol–Xhol-digested pHueScript, creating pNde-TLG2aΔTM.

For bacterial expression, the NdeI–Xhol fragment of pNde-TLG2aΔTM was cloned into similarly digested pET-23b (Novagen). This plasmid resulted in a translational fusion of amino acids 1–299 of AtTLG2a with six histidine residues. This plasmid was introduced into Escherichia coli BL21 (DE3) pLysS cells, and expression of AtTLG2a(1–299)6xHis was induced by isopropylthio-β-galactoside. Overexpressed protein was purified by nickel affinity chromatography as described by the manufacturer (Novagen) and used to immunize a rabbit at Cocalico Biologicals (PA).

The antiserum was subjected to affinity purification (Bar-Peled and Raikhel, 1996) with the use of E. coli–expressed maltose-binding protein fusions of AtTLG2a chemically cross-linked to amyllose resin. A translational fusion of amino acids 1–299 of AtTLG2a to the C-terminus of maltose-binding protein was created by cloning the Xhol–Xhol fragment of pNde-TLG2aΔTM into Xhol–SalI-digested pMALc2 (New England Biolabs, Beverly, MA). E. coli BL21 cells bearing these plasmids were induced with isopropylthio-β-galactoside, and overexpressed protein was bound batchwise to amyllose affinity columns as described by the manufacturer (New England Biolabs). The amyllose columns containing bound protein were equilibrated with 100 mM sodium borate, pH 9.0, before dimethyl pemilimidate was added to a final concentration of 4 mg/ml. After incubation at room temperature for 30 min, excess cross-linker was washed with 100 mM ammonium chloride and by several washes in 1 M Tris-HCl, pH 8.0. Columns were stripped of non-cross-linked protein with washes in 100 mM glycine, pH 2.5, and then equilibrated with PBS. Crude serum raised to AtTLG2a was applied to the column. The column was washed extensively with PBS containing 0.05% Tween-20, and bound antibodies were eluted stepwise with 100 mM glycine, pH 2.5. After neutralization, antibody-containing fractions were pooled, dialyzed against PBS, concentrated to >2 mg/ml (Millipore [Bedford, MA] Ultrafree Biomax-5K), and then stored in PBS containing 1% BSA and 0.02% azide.
Production of Antiserum against AtVTI1b

To produce protein in bacteria for use in generating antibodies, the AtVTI1b cDNA was modified by PCR to generate a BamHI restriction site at the start of the ORF and a SalI restriction site just before the transmembrane domain (VTI1b-F: AAG GAT CCA GAT GAG CGA CCT ATT TGA A; VTI1b-R: AAG TCG ACA TTT GTT CCT AGT CAT TCT). The PCR fragment was cloned into BamHI–SalI-digested pET-14b (Novagen). Recombinant His-tagged AtVTI1b was overexpressed and purified with the use of a Ni²⁺ column according to the manufacturer’s protocol (Novagen) and used to immunize a rabbit at Cocalico Biologicals to produce antibodies against AtVTI1b.

Expression of Epitope-tagged AtTLG2a and AtTLG2b in Arabidopsis

AtTLG2a containing an N-terminal hemagglutinin (HA) epitope was constructed as follows: pNde-TLG2a was digested with NdeI and Xhol and cloned into similarly digested pACT-2 (Clontech, Palo Alto, CA), resulting in fusion of the Gal4 activation domain and an HA epitope to the N terminus of AtTLG2a. Digestion of this plasmid with BglII and Xhol released a fragment that encoded for a start codon, the HA epitope, and the full-length AtTLG2a, which was used in subsequent studies. AtTLG2b was tagged with a T7 epitope as follows: pNdeTLG2b was digested with Xhol, followed by treatment with T4 DNA polymerase (Boehringer Mannheim, Indianapolis, IN) in the presence of deoxynucleoside triphosphates, heat treatment to inactivate the polymerase, and then digestion with Xhol. This DNA fragment was cloned into pET-23b (Novagen) that was digested with BamHI, followed by treatment with T4 DNA polymerase (Boehringer Mannheim) in the presence of deoxynucleoside triphosphates, heat treatment to inactivate the polymerase, and then digestion with Xhol, resulting in a fusion of the T7 epitope to the N terminus of AtTLG2b. The fusions were confirmed by sequencing and by expression of each fusion in either bacteria or yeast cells. HA-AtTLG2a and T7-AtTLG2b were inserted behind the constitutive cauliflower mosaic virus 35S promoter and introduced into A. thaliana ecotype Columbia by vacuum infiltration (Bent et al., 1994). Plants were screened for antibiotic resistance, and expression of the epitope-tagged proteins was confirmed with the use of HA polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA) or T7 monoclonal (Novagen) antibodies.

Expression of HA-tagged AtVTI1b in Arabidopsis

The AtVTI1b cDNA (Zheng et al., 1999b) was modified by PCR with the use of a 3′ primer that coded for the nine-amino acid HA tag (AAG GAT CCA GAT GAG CGA CCT ATT TGA A; VTI1b-F: AAG TCG ACA TTT GTT CCT AGT CAT TCT) with a 5′ primer that coded for the nine-amino acid HA tag (AAG GAT CCA GAT GAG CGA CCT ATT TGA A; VTI1b-R: AAG TCG ACA TTT GTT CCT AGT CAT TCT). The PCR fragment was cloned into BamHI–SalI-digested pET-14b (Novagen). Recombinant His-tagged AtVTI1b was overexpressed and purified with the use of a Ni²⁺ column according to the manufacturer’s protocol (Novagen) and used to immunize a rabbit at Cocalico Biologicals to produce antibodies against AtVTI1b.

Immunoprecipitation of Detergent Extracts of Arabidopsis Roots

AtVTI1b antibodies were affinity purified against E. coli–synthesized AtVTI1b as described by Bassham and Raikhel (1998). The purified antibodies were covalently linked to protein A–Sepharose beads with the use of dimethylpimelimidate according to Harlow and Lane (1988). Four-week-old Arabidopsis plants grown in liquid culture (Bar-Peled et al., 1995) were ground with a mortar and pestle in extraction buffer (0.3 M sucrose, 0.1 M Tris–HCl, pH 7.5, 1 mM EDTA, 0.1 mM PMSF) and passed through Miracloth (Calbiochem, La Jolla, CA) to remove debris. The extract was centrifuged at 1000 × g, and the supernatant was further centrifuged at 100,000 × g to generate a membrane pellet. The pellet was resuspended in PBS containing 1% (vol/vol) Triton X-100, 10 mM β-mercaptoethanol, and protease inhibitors and rocked for 2 h at 4°C to solubilize membrane proteins. Insoluble material was pelleted at 100,000 × g, and the supernatant (total fraction) was incubated overnight with the antibody column, rocking at 4°C. The beads were allowed to settle, and the unbound protein (flow-through fraction), including any protein aggregates, was removed. The beads were washed five times with PBS containing 1% Triton X-100, and bound protein was eluted with the use of 100 mM glycine, pH 2.5 (eluate fraction). Equal volumes of the total and flow-through fractions (equivalent to one-twentieth of the eluate) and the entire eluate fraction were analyzed by immunoblotting with antibodies against AtVPS45, AtELP, or various Arabidopsis SNAREs. The entire eluate fraction was loaded to allow the detection of proteins that interact with AtVPS45 only transiently or nonstochiometrically and that therefore may be present in very low amounts.

AtTLG2a and T7-AtTLG2b were immunoprecipitated from detergent extracts of either wild-type or T7-AtTLG2b–expressing roots grown in liquid culture. Protein A–Sepharose beads with bound affinity-purified antibodies to AtTLG2a or immobilized T7 mAbs (Novagen) were incubated with detergent extracts prepared as described above for 2 h at 4°C. The flow through was collected and precipitated with 5% trichloroacetic acid, followed by resuspension in SDS sample buffer (188 mM Tris, pH 6.8, 30% [vol/vol] glycerol, 5% [wt/vol] SDS, 5% [vol/vol] β-mercaptoethanol). Beads were washed in PBS containing 1% (vol/vol) Triton X-100 and then eluted in SDS sample buffer. Equivalent amounts of flow through and eluate were separated by SDS-PAGE and analyzed by immunoblotting.

RESULTS

AtVPS45 Is Localized to the TGN in Arabidopsis Roots

On sucrose density gradients, AtVPS45 has been shown to cofractionate with the cargo receptor AtELP and not with the prevacuolar t-SNARE AtPEP12 (Bassham and Raikhel, 1998). To provide insight into the potential function of AtVPS45, we wanted to identify the compartment on which AtVPS45 resides. When conventional electron microscopic fixation and sectioning techniques were used, no immunogold labeling could be detected with AtVPS45 antibodies. Therefore, immunogold electron microscopy was performed on cryosections of Arabidopsis roots with the use of affinity-purified antibodies raised against AtVPS45 (Bassham and Raikhel, 1998). The cryosectioning technique maintains protein antigenicity much better than conventional techniques and therefore allows the detection of the membrane proteins under study. However, the membrane structure is not as well preserved, so the morphology of organelles is sometimes not as clear. As seen in Figure 1, gold particles specifically labeled a network of membrane structures at the trans side of the Golgi apparatus (Figure 1A) and the dilated ends of the trans cisternae (Figure 1B). Very little labeling was seen over any other organelle. In control samples, no labeling was seen when preimmune serum was used (Figure 1C).

To determine more precisely the identity of the membranes with which AtVPS45 associates, double-labeling experiments were performed with the use of antibodies against AtVPS45 and either AtELP or AtPEP12 (Figure 2). AtELP is a potential vacuolar cargo receptor that resides on the PVC, where it colocalizes with the t-SNARE AtPEP12,
and on the TGN (Sanderfoot et al., 1998). Immunogold electron microscopy with antibodies against AtVPS45 (10 nm gold; arrows) and AtELP (5 nm gold; arrowheads) revealed that at least one population of each protein resides on the same membrane (Figure 2A). The structures labeled by both antibodies appear to be always closely associated with the Golgi apparatus, and in sections in which the orientation of the Golgi can be clearly identified, they are found at the trans side and therefore are likely to be the TGN. In some sections, the two antibodies labeled separate Golgi-associated structures (Figure 2B; Table 1); it is not clear whether this is due to technical limitations or whether a subpopulation of AtVPS45 does in fact reside on a different membrane or membrane domain than AtELP. Controls were used to ensure the specificity of the double immunolabeling, consisting of anti-AtELP (5 nm gold; Figure 2C, arrowheads) with AtVPS45 preimmune serum (10 nm gold) and anti-AtVPS45 (10 nm gold; Figure 2D, arrow) with AtELP preimmune serum (5 nm gold). In each case, the preimmune serum gave no labeling.

To confirm that AtVPS45 colocalizes with the TGN pool of AtELP, and not the pool at the prevacuolar compartment, double immunogold labeling was performed with antibodies against the t-SNARE AtPEP12. AtPEP12 resides exclusively on the prevacuolar compartment, and AtPEP12 antibodies were shown previously to specifically label this compartment (Conceição et al., 1997; Sanderfoot et al., 1999). When AtVPS45 antibodies (10 nm gold; Figure 2E, arrows) and AtPEP12 antibodies (5 nm gold; arrowheads) were used in double-labeling experiments, the gold particles always labeled separate organelles (Table 1), with no colocalization seen. AtVPS45 labeling was found associated with the Golgi, whereas AtPEP12 labeling was over electron-dense structures often more distant from the Golgi, as expected for the PVC. This confirms that AtVPS45 is not found on the PVC but rather is localized to the TGN in Arabidopsis roots. Again, controls consisting of anti-AtPEP12 (5 nm gold) with AtVPS45 preimmune serum (10 nm gold; Figure 2F) and anti-AtVPS45 (10 nm gold) with AtPEP12 preimmune serum (5 nm gold; Figure 2G) demonstrated the specificity of the labeling.

**AtVPS45p Can Bind to Arabidopsis Tlg2p Homologues**

AtVPS45 encodes a Sec1p-like protein from Arabidopsis (Bassham and Raikhel, 1998). Proteins of the Sec1p family are known to interact with a particular t-SNARE at the target membrane as part of their function in vesicle fusion. Therefore, it is predicted that AtVPS45 should interact with one or more t-SNAREs that function at the same step of vesicle transport. To determine whether AtVPS45 can interact with t-SNAREs from Arabidopsis, we chose to use yeast as an expression system for the Arabidopsis proteins, because mutant complementation experiments indicate that the proteins are functional in this system and therefore able to fold correctly. Five different potential Arabidopsis endomembrane t-SNAREs were used in binding assays, as follows. AtPEP12 (Bassham et al., 1995) and AIVAM3 (Sato et al., 1997) are both homologous to yeast Pep12p and have been localized to the PVC in Arabidopsis roots (Conceição et al., 1997; Sanderfoot et al., 1999). AtSED5 shows high sequence similarity to the yeast cis-Golgi t-SNARE Sed5p (Hardwick and Pelham, 1992; see MATERIALS AND METHODS). AtTLC2a and -b are two newly isolated Arabidopsis t-SNAREs that are highly similar to each other (68% amino acid identity) as well as to yeast Tlg2p. Each t-SNARE was fused to a six-histidine tag and coexpressed in yeast with untagged AtVPS45. Expression levels were similar for each protein, as determined by immunoblotting (Figure 3). The yeast cells were lysed, membranes were solubilized with nonionic detergent, and the
Figure 2. AtVPS45 colocalizes with AtELP, and not with AtPEP12, on ultrathin cryosections of Arabidopsis roots. (A and B) Double labeling for AtELP and AtVPS45. Sections were incubated with AtELP antibodies followed by biotinylated goat anti-rabbit secondary antibodies and detected with the use of streptavidin conjugated to 5-nm gold particles. After a second fixation step, the same sections were incubated with antibodies against AtVPS45, then biotinylated goat anti-rabbit antibodies, and detected with the use of streptavidin conjugated to 10-nm gold particles. (C) As for A and B, except preimmune serum was substituted for the AtVPS45 antibodies. (D) As for A and B, except preimmune serum was substituted for AtELP. (E) Double labeling for AtPEP12 and AtVPS45. Sections were labeled according to the protocol described in A and B, with the use of AtPEP12 antibodies followed by 5-nm gold particles and AtVPS45 antibodies followed by 10-nm gold particles. (F) As for E, except preimmune serum was substituted for AtVPS45 antibodies. (G) As for E, except preimmune serum was substituted for AtPEP12 antibodies. G, Golgi. Arrows, 10-nm gold particles (labeling AtVPS45); arrowheads, 5-nm gold particles (labeling AtELP or AtPEP12). Bar, 0.1 μm.
His-tagged t-SNAREs were affinity purified with the use of a Ni\(^{2+}\)-agarose column. Bound protein was eluted with the use of imidazole and analyzed by immunoblotting with antibodies against the histidine tag or AtVPS45 (Bassham and Raikhel, 1998). In each case, the His-tagged t-SNARE bound to the Ni\(^{2+}\)-agarose column and was eluted with imidazole. AtVPS45 was seen to bind only to the t-SNAREs AtTLG2a and AtTLG2b (Figure 3). No AtVPS45 copurified with AtPEP12 or AtVAM3, the prevacuolar t-SNAREs (Conceição et al., 1997; Sanderfoot et al., 1999), or with the AtSED5 control. This suggests that, at least in this heterologous system, AtVPS45 cannot interact with AtPEP12 but instead interacts with two different Tlg2p-like proteins from *Arabidopsis*.

**AtTLG2a, AtTLG2b, and AtVTI1b Coimmunoprecipitate with AtVPS45 from *Arabidopsis* Extracts**

To study AtTLG2a in vivo, we raised antibodies to the cytosolic domain of AtTLG2a in rabbits. After affinity purification, this antiserum was found to recognize a series of bands from *Arabidopsis* microsomal extracts (Figure 4A, lane 1): three major bands of ~39, 36, and 34 kDa and a minor band of 44 kDa. Expression of the *AtTLG2a* cDNA in yeast confirmed that the three major bands are derived from this cDNA. The 39-kDa band (marked with an arrowhead) represents the full-length AtTLG2a, whereas the lower two bands are probably breakdown products because the presence and intensity of these bands in both yeast and plants were found to vary depending on the presence of protease inhibitors. Because expression of AtTLG2b in yeast yielded a protein of ~44 kDa that cross-reacted with the AtTLG2a antibodies, it is likely that the very weakly reacting band in *Arabidopsis* microsomes of 44 kDa corresponds to AtTLG2b. As yet, we have been unable to generate antibodies specific to AtTLG2b. To clearly differentiate between AtTLG2a and AtTLG2b, each protein was epitope tagged at the N terminus: AtTLG2a with an HA epitope and AtTLG2b with a T7 epitope. The T7 tag has been used successfully to epitope tag plant SNAREs (Sanderfoot et al., 1999; Zheng et al., 1999b) with no effect on their subcellular localization or function. *Arabidopsis* plants were transformed with these constructs, and plants expressing HA-AtTLG2a and T7-

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For each combination of proteins shown, the number of Golgi stacks in which colocalization was seen (Together; defined as within 80 nm), with antibodies against epitope-tagged or endogenous proteins, and in which the labeling was seen over different membranes or membrane domains (Separate), is indicated. The total number of Golgi stacks examined for each combination is indicated in the Total column.

**Table 1.** Quantitation of immunogold electron microscopy double labeling.

**Figure 3.** AtVPS45 interacts with AtTLG2a and AtTLG2b in a yeast expression system. Yeast were cotransformed with cDNAs encoding the His-tagged t-SNAREs AtSED5, AtPEP12, AtTLG2a, AtVAM3, or AtTLG2b, or with vector alone as a control, and untagged AtVPS45. The t-SNAREs were isolated from the yeast extracts with the use of a Ni\(^{2+}\)-agarose column, and aliquots of the total extracts (TOTAL) and purified t-SNARE complexes (BOUND) were analyzed by SDS-PAGE and immunoblotting with antibodies against AtVPS45.

**Figure 4.** Characterization of antibodies and epitope tags for AtTLG2a/b and AtVTI1a/b. (A) A microsomal extract of wild-type *Arabidopsis* (lane 1) was probed with affinity-purified antiserum to AtTLG2a. Full-length AtTLG2a is indicated by the arrowhead, and the smaller bands represent proteolytic breakdown products (see text). Antiserum to AtTLG2a cross-reacts weakly with AtTLG2b, which is indicated by the asterisk. Total protein extracts from either wild type (lanes 2 and 5) or plants expressing either HA-AtTLG2a (lanes 3 and 6) or T7-AtTLG2b (lanes 4 and 7) were probed with antisera specific to the epitope tags. Lanes 2–4 were probed with rabbit anti-HA to indicate HA-AtTLG2a, whereas lanes 5–7 were probed with mouse T7 mAbs to indicate T7-AtTLG2b. (B) Total extracts of wild-type *Arabidopsis* (lanes 1 and 4), plants expressing T7-AtVTI1a (lanes 2 and 5), or plants expressing HA-AtVTI1b (lanes 3 and 6) were probed with rabbit polyclonal antisera raised to either AtVTI1a (lanes 1–3) or AtVTI1b (lanes 4–6). In each case, the endogenous AtVTI1a or AtVTI1b is indicated by the arrowhead, and the epitope-tagged protein is indicated by the asterisk.
AtTLG2b were recovered. Each of the epitope-tagged proteins migrated at the expected size on SDS-PAGE after detection with appropriate antisera: HA-AtTLG2a migrated as a band of 38 kDa (Figure 4A, lane 3), and T7-AtTLG2b migrated at 45 kDa (Figure 4A, lane 7). No obvious phenotypic differences were observed in these plants, suggesting that heterologous expression of these tagged t-SNAREs had no detrimental effect.

To investigate further the interaction between AtVPS45 and t-SNAREs in vivo, AtVPS45 was immunoprecipitated from detergent extracts of microsomes from *Arabidopsis* with the use of affinity-purified AtVPS45 antibodies, and the presence of various proteins in the immunoprecipitate was determined by immunoblotting (Figure 5). As expected, AtVPS45 was found in the immunoprecipitate, whereas the vacuolar cargo receptor AtELP was not. Consistent with the binding experiments with proteins expressed in yeast, AtTLG2a coprecipitated with AtVPS45, whereas AtPEP12 and AtVAM3 did not. This implies that AtVPS45 and AtTLG2a interact in vivo and that the two proteins probably function in the same vesicle transport step.

To determine whether AtTLG2b also coprecipitates with AtVPS45 antibodies, we used transgenic *Arabidopsis* expressing AtTLG2b fused to a T7 epitope tag. The immunoprecipitate was probed with antibodies against the T7 epitope tag. These antibodies detected T7-AtTLG2b in the precipitate, confirming that AtTLG2b can also interact with AtVPS45 in *Arabidopsis*.

To test for the coprecipitation of other *Arabidopsis* SNAREs with AtVPS45, antibodies were also raised against AtVTI1b (Zheng et al., 1999b). *Arabidopsis* has two proteins (AtVTI1a and AtVTI1b) that are both similar to the yeast SNARE Vti1p, which is required for multiple transport steps, including several different routes to the vacuole. Using different yeast mutant alleles, we demonstrated previously that AtVTI1a can function in the carboxypeptidase Y pathway, whereas AtVTI1b can function in the alkaline phosphatase and Cvt pathways to the vacuole (Zheng et al., 1999b). Antibodies raised against AtVTI1a (Zheng et al., 1999b) are specific to that isoform, but antibodies raised against recombinant AtVTI1b recognize AtVTI1a as well as AtVTI1b (Figure 4B).

Each of these antisera was used to probe the AtVPS45 immunoprecipitate (Figure 5). Very little AtVTI1a could be detected in the immunoprecipitate, although it could be detected in the total protein sample. To confirm this result, AtVPS45 immunoprecipitation was repeated with the use of transgenic *Arabidopsis* plants producing a T7-tagged AtVTI1a (Zheng et al., 1999b) and the immunoprecipitate was probed with T7 mAbs. Again, very little T7-AtVTI1a was present in the precipitate, confirming that AtVTI1a does not interact with AtVPS45. In contrast, the AtVTI1b antibodies detected a band in the immunoprecipitate that corresponds to AtVTI1b. AtVPS45 antibodies were also able to coimmunoprecipitate HA-tagged AtVTI1b from transgenic plants expressing this protein, confirming that the precipitated protein is AtVTI1b. In both cases, the amount of AtVTI1b precipitated was only a small percentage of the total amount of AtVTI1b in the protein extract. We conclude that some AtVTI1b, albeit a small amount, is found in a complex with AtVPS45 in *Arabidopsis* roots, whereas AtVTI1a is not. This is the first evidence in plants that the two *Arabidopsis* Vti1p-like proteins are involved in distinct pathways and do not have redundant functions.

Because the amount of AtVTI1b present in the immunoprecipitate was very small, we wanted to confirm by independent means that AtVTI1b, and not AtVTI1a, is found in complex with AtVPS45. Therefore, we decided to immunoprecipitate the AtVPS45 complex with antibodies against AtTLG2a (Figure 5B). As expected, AtTLG2a antibodies coimmunoprecipitated AtVPS45 and not AtELP. Similar to the AtVPS45 precipitations, AtVTI1b was coprecipitated to a much greater extent than AtVTI1a, although still a very small amount compared with the total extract. The same result was obtained when T7-AtTLG2b was immunoprecipitated from transgenic plants with T7 antibodies (Figure 5C). Thus, it seems likely that AtVTI1b, and not AtVTI1a, is present in complexes containing AtVPS45 and AtTLG2a or -b.

**AtTLG2a and AtTLG2b Are Present in Different Domains of the TGN**

The interaction between AtVPS45 and both AtTLG2a and AtTLG2b suggests that the three proteins may be found on the same membrane. To determine the subcellular location of AtTLG2a, immunogold electron microscopy was performed on *Arabidopsis* roots with the use of AtTLG2a antibodies (Figure 6A; 10 nm gold). The gold particles labeled membranes at the trans side of the Golgi, with a labeling pattern strongly resembling that of the AtVPS45 antibodies. AtTLG2a, therefore, is likely to reside on the TGN. No labeling was seen over any other organelle, and no labeling was seen with preimmune serum (Figure 6B). The antibodies against AtTLG2a cross-re-
Figure 6. AtTLG2a and AtTLG2b localize to different subdomains of the TGN. (A) Ultrathin cryosections of Arabidopsis roots were labeled with AtTLG2a antibodies followed by biotinylated goat anti-rabbit secondary antibodies and detected with the use of streptavidin conjugated to 10-nm gold particles. (B) Control section probed as for A except for the use of preimmune serum in place of the AtTLG2a antibodies. (C) Sections from transgenic plants expressing T7-AtTLG2b were incubated with T7 mAbs followed by a rabbit anti-mouse bridge, biotinylated goat anti-rabbit antibodies, and streptavidin conjugated to 5-nm gold particles. (D) Control section as for C except incubated with 2% BSA in place of the T7 mAbs. (E) Sections from transgenic Arabidopsis producing HA-AtTLG2a and T7-AtTLG2b were incubated with HA polyclonal antibodies followed by biotinylated goat anti-rabbit secondary antibodies and detected with the use of streptavidin conjugated to 10-nm gold particles. After a second fixation step, the same sections were incubated with T7 mAbs, followed by a rabbit anti-mouse bridge and then biotinylated goat anti-rabbit antibodies, and detected with the use of streptavidin conjugated to 5-nm gold particles. (F) Double control probed as for E except for the use of nonimmune serum in place of HA antibodies and 2% BSA in place of T7 antibodies. G, Golgi. Arrow, 10-nm gold particles (labeling HA-AtTLG2a); arrowheads, 5-nm gold particles (labeling T7-AtTLG2b). Bar, 0.1 μm.
acted very weakly on an immunoblot with AtTLG2b; therefore, it was considered likely that the labeling seen by immunoelectron microscopy is due to AtTLG2a and not AtTLG2b. Sections from plants expressing HA-tagged AtTLG2a were used in double-labeling experiments with AtTLG2a antibodies and HA antibodies, and the two labels indicated that the endogenous and heterologous proteins colocalize. Immunogold labeling was also performed on root tissue from transgenic Arabidopsis plants producing T7-AtTLG2b with antibodies against the T7 tag (Figure 6C; 5 nm gold). Again, labeling appears to be at the TGN, in a pattern similar to that described for AtVPS45, and no labeling was seen in control sections (Figure 6D). Double-labeling of transgenic plants producing HA-tagged AtTLG2a and T7-tagged AtTLG2b with HA antibodies (10 nm gold) and T7 antibodies (5 nm gold) was used to determine whether AtTLG2a and AtTLG2b are present on the same membrane (Figure 6E). Unexpectedly, although both antibodies label what appears to be the TGN, colocalization of the two proteins was almost never seen (Table 1). Therefore, it is likely that AtTLG2a and AtTLG2b are found in different domains of the TGN and probably have distinct functions in vesicle trafficking. The same results were observed when sections were probed with AtTLG2a antibodies and T7 antibodies. Control sections were free of label (Figure 6F).

To characterize further the differential distribution of AtTLG2a and -b on the TGN, double immunogold labeling was performed on these transgenic plants with antibodies against the T7 or HA tags and AtELP (Table 1). HA-AtTLG2a and T7-AtTLG2b both colocalized on some Golgi stacks with AtELP. Interestingly, AtELP appeared to colocalize more frequently with AtTLG2b than with AtTLG2a, although it is not yet clear whether this is significant or whether it reflects a functional difference between the two t-SNAREs.

To definitively distinguish between staining of the TGN and the PVC, double immunogold labeling was carried out with the use of antibodies against AtTLG2a (10 nm gold) and AtPEP12 (5 nm gold; Figure 7A and inset, arrowheads) labeling was seen in separate parts of the Golgi and TGN. However, antibodies to both proteins labeled the same membranes in ~50% of sections (Figure 7B and inset), which we conclude is the TGN. Cross-reactivity of the secondary antibodies was ruled out by controls with the use of anti-AtTLG2a (10 nm gold) with AtVPS45 preimmune serum (15 nm gold; Figure 8C) and anti-AtVPS45 (10 nm gold) with AtTLG2a preimmune serum (5 nm gold; Figure 8D). In addition, when preimmune sera from both AtVPS45 (10 nm gold) and AtTLG2a (5 nm gold) were used, no labeling could be detected (Figure 8E). Therefore, it appears that at least a portion of the AtVPS45 and AtTLG2a in a cell resides on the same organelle. Similar double-labeling experiments were performed with the use of AtVPS45 (10 nm gold) and T7-AtTLG2b (5 nm gold). Controls were similar to those described above. As for AtTLG2a, in ~50% of sections AtVPS45 and T7-AtTLG2b colocalized at the TGN (Figure 8, G and H), but in the remainder they were found separately (Figure 8F).

**AtTLG2a and AtTLG2b Colocalize with AtVPS45**

To determine whether AtTLG2a is found in the same organelle as AtVPS45, double immunogold labeling was again performed. In some sections, AtTLG2a (5 nm gold; Figure 8A and inset, arrowheads) and AtVPS45 (10 nm gold; arrows) labeling was seen in separate parts of the Golgi and TGN. However, antibodies to both proteins labeled the same membranes in ~50% of sections (Figure 8B and inset), which we conclude is the TGN. Cross-reactivity of the secondary antibodies was ruled out by controls with the use of anti-AtTLG2a (10 nm gold) with AtVPS45 preimmune serum (15 nm gold; Figure 8C) and anti-AtVPS45 (10 nm gold) with AtTLG2a preimmune serum (5 nm gold; Figure 8D).
Figure 8. AtTLG2a and AtTLG2b colocalize with AtVPS45. Double labeling was performed as described in Figure 2. (A and inset) AtTLG2a and AtVPS45 on separate parts of the TGN. Cryosections were labeled with AtTLG2a antibodies (5 nm gold) followed by AtVPS45 antibodies (10 nm gold). (B) AtTLG2a and AtVPS45 sometimes colocalize at the TGN. Sections were labeled with AtTLG2a antibodies (10 nm gold) followed by AtVPS45 antibodies (15 nm gold). (B inset) Sections were labeled with AtTLG2a antibodies (5 nm gold) followed by AtVPS45 antibodies (10 nm gold). (C) As for B except AtVPS45 preimmune serum was used in place of anti-AtVPS45. (D) As for A except AtTLG2a preimmune serum was used in place of anti-AtTLG2a. (E) Double control as for A but with both preimmune sera in place of the equivalent antibody. (F–H) Sections from transgenic Arabidopsis expressing T7-tagged AtTLG2b. Labeling was performed as described in Figure 6. (F) T7-AtTLG2b and AtVPS45 on separate parts of the TGN. Sections were labeled with T7 antibodies (5 nm gold) followed by AtVPS45 antibodies (10 nm gold). (G and H) T7-AtTLG2b and AtVPS45 sometimes colocalize at the TGN. Sections were labeled with T7 antibodies (5 nm gold) followed by AtVPS45 antibodies (10 nm gold). G, Golgi. Arrows, 10-nm gold particles (15 nm in B); arrowheads, 5-nm gold particles (10 nm in B). Bar, 0.1 μm.
AtVPS45 is a member of this protein family from various organelles of the secretory pathway. The Sec1p family of proteins is involved in vesicle trafficking.

**DISCUSSION**

The Sec1p family of proteins is involved in vesicle trafficking between various organelles of the secretory pathway. AtVPS45 is a member of this protein family from Arabidopsis that was isolated previously (Bassham and Raikhel, 1998) based on its sequence similarity to yeast Vps45p, a Sec1p-like protein required for transport to the vacuole (Cowles et al., 1994; Piper et al., 1994). Based on the vps45 mutant phenotype and the interaction of yeast Vps45p with the prevacuolar t-SNARE Pep12p, Vps45p has been proposed to reside at the PVC in yeast. AtVPS45 complements two different phenotypes of the yeast vps45 mutant (Bassham and Raikhel, 1998), suggesting that AtVPS45 can functionally replace yeast Vps45p. However, sucrose density gradient analysis of membranes from Arabidopsis roots indicated that AtVPS45 does not cofractionate with AtPEP12 and thus may not be present on the PVC (Bassham and Raikhel, 1998).

Mammalian Vps45p-like proteins have also been identified (Pevsner et al., 1996; Tellam et al., 1997) based on sequence similarity to yeast Vps45p. Although they have been proposed to play a role in vesicle transport to the lysosome, their function has not yet been demonstrated. Mammalian VPS45 is unable to complement the yeast vps45 mutant, and immunofluorescence microscopy showed localization to the Golgi/TGN (Bock et al., 1997; Tellam et al., 1997). These apparent inconsistencies between species demonstrate that it is critical to study the localization and function of Vps45p-like proteins in multicellular organisms rather than simply extrapolate from yeast as a model system.

As a first step to understanding the function of AtVPS45 in plant cells and, more generally, the physiological role of Vps45p-like proteins in multicellular organisms, we investigated its subcellular localization in more detail. AtVPS45 has now been shown to reside on the Golgi apparatus, and in particular is concentrated at the TGN, as defined morphologically by electron microscopy and by colocalization with the vacuolar cargo receptor AtELP. The distinct locations of AtVPS45 and AtELP in some sections may be readily explained, because AtELP is an itinerant protein that is packaged into clathrin-coated vesicles at the TGN for transport to the PVC, and AtVPS45 is unlikely to be involved in this process, because all known members of the Sec1p family appear to function in membrane fusion. The AtELP present in budding vesicles thus would not be expected to colocalize with AtVPS45. No AtVPS45 labeling can be observed at the PVC, defined by the presence of the t-SNARE AtPEP12. In addition, AtVPS45 fails to interact with the prevacuolar t-SNARE AtPEP12 in a heterologous yeast expression assay or by immunoprecipitation from root tissue. It appears likely that, unlike in yeast, AtPEP12 and AtVPS45 function in distinct transport steps and at different organelles. If this is the case, an intriguing question remains to be addressed: Does another, as yet unidentified, Vps45p-like protein exist in Arabidopsis that interacts with AtPEP12 and functions in the fusion of TGN-derived transport vesicles with the PVC? Searches of the sequence databases have not revealed an additional closely related Vps45p homologue, and a final answer to this question awaits the completion of the genome sequence of Arabidopsis. However, genes encoding several putative members of the Sec1p family can be identified, including a potential Vps33p homologue and several proteins most related to Sec1p itself. Yeast Vps33p is required for transport from the PVC to the vacuole (Banta et al., 1990), and it will be interesting to determine whether the Arabidopsis homologue also functions in this step or whether, like AtVPS45, there are differences in function and localization between yeast and plants. The presence of several Sec1p homologues in Arabidopsis is also intriguing. This may reflect simple redundancy in secretory pathway function or specialization in different tissues or developmental stages.

The association of AtVPS45 with the TGN implies that it must interact with an integral membrane protein at the TGN to associate with the membrane. Several such interactions have now been described. AtVPS45 interacts with AtTLG2a and AtTLG2b, syntaxin-like proteins from Arabidopsis with similarity to yeast Tlg2p, and also with a third SNARE.
AtVTI1b. The subcellular location of Tlg2p in yeast remains somewhat equivocal, with reports of its presence on the late Golgi (Abeliovich et al., 1998; Holthuis et al., 1998), early endosomes (Séron et al., 1998; Lewis et al., 2000), or late endosomes (equivalent to the PVC; Abeliovich et al., 1998). The mammalian Tlg2p homologue, Syntaxin 16, has been reported to localize to the Golgi (Simonsen et al., 1998; Tang et al., 1998). In Arabidopsis, we have demonstrated that AtTLG2a and AtTLG2b reside on the TGN, where they colocalize with AtVPS45, as expected for associated proteins. However, AtTLG2a and AtTLG2b do not colocalize by immunogold electron microscopy but instead reside on distinct regions of the TGN. This suggests that AtVPS45 forms separate complexes with AtTLG2a and AtTLG2b and that these complexes may define functional domains of the Arabidopsis TGN. An intriguing difference is seen in the extent of colocalization of each with AtELP. Whereas both AtTLG2a and -b are sometimes found on the same membrane (or membrane region) as AtELP, AtELP is most often seen in close proximity to AtTLG2b. Whether this reflects a functional difference remains to be seen.

Interestingly, one other SNARE was found to coprecipitate with AtVPS45: AtVTI1b, which is also a member of a small family of proteins in Arabidopsis (Zheng et al., 1999b). In Arabidopsis, two homologues of yeast Vti1p are found, AtVTI1a and -b. We have shown previously that AtVTI1a is able to suppress the carboxypeptidase Y–sorting defect of yeast vti1 mutant alleles and that, in Arabidopsis, it interacts with the PVC t-SNAREs AtPEP12 and AtVM3 (Sanderfoot et al., 1999; Zheng et al., 1999b), suggesting that AtVTI1a acts in a TGN-to-PVC targeting pathway. Consistent with our findings that AtVPS45 does not appear to function in the fusion of vesicles at the PVC, AtVTI1a is not coprecipitated by AtVPS45. On the other hand, AtVTI1b could not suppress the carboxypeptidase Y–sorting defects and instead was found to suppress defects in Cvt sorting that occur in vti1 mutant alleles. Our finding that AtVTI1b appears to enter the AtVPS45-AtTLG2a or -b complex is the first indication in plants that members of the AtVTI1 family may play separate roles in transport reactions, reflecting their distinct behavior when expressed in yeast. Therefore, we have identified two yeast proteins (Tlg2p and Vti1p) that have multiple isoforms in Arabidopsis with nonredundant functions. In Arabidopsis, other compartments of the endomembrane system have also been found to contain multiple syntaxin-like t-SNAREs that have a high degree of sequence similarity. For example, at least two other genes encoding proteins with similarity to AtPEP12 exist: AtVM3, which is known to be colocalized with AtPEP12 on the PVC in roots (Sanderfoot et al., 1999), and AtPLP, the subcellular location of which is unknown (Zheng et al., 1999a). The elucidation of the function of each member of each family is critical to our understanding of the complex endomembrane system of plant cells.

Sec1p was recently shown to be capable of interacting with an intact SNARE complex (Carr et al., 1999), which leads to the possibility that AtVPS45 is in fact interacting with a SNARE complex containing both AtTLG2a or -b and AtVTI1b. However, it is not possible to determine from these data whether the interaction is direct or whether all three proteins are present in a single complex. AtVTI1a and -b are present on the TGN, and both colocalize to some extent with AtVPS45, confirming that a complex containing AtVPS45, AtTLG2a or -b, and AtVTI1b is possible in vivo. A similar result has been found in yeast, in which antibodies against the t-SNARE Tlg1p can coprecipitate Vps45p, Tlg2p, and Vti1p (Coe et al., 1999). In this case, it is also unknown whether the proteins are all present in the same complex or in a number of subcomplexes. Analysis of these findings is complicated because the exact role of nontypical SNAREs such as Vti1p and its homologues in other eukaryotes remains unclear. Although Vti1p was originally referred to as a v-SNARE, structural and sequence features best describe it as a Q-SNARE, allied more with members of the syntaxin and SNAP-25 families (also Q-SNAREs), as opposed to typical v-SNAREs such as yeast Snc1p and mammalian synaptobrevins (referred to as R-SNAREs in this context; Fasshauer et al., 1999). Thus, the presence of Vti1p (or AtVTI1b) in these complexes may simply represent a multicomponent t-SNARE complex, rather than a classic “four-helix bundle” SNARE complex such as that found in the mammalian neuron (Sutton et al., 1998). Further analysis will be required to answer these questions.

In light of the data presented here, the function of AtVPS45 in vesicular transport en route to the vacuole needs to be reevaluated. In all transport steps through the secretory pathway studied in detail thus far, a Sec1-like protein appears to be involved and functions in a membrane fusion reaction. The basic biochemical function of Sec1-like proteins, although currently unclear, is likely to be conserved among different species. Therefore, we conclude that AtVPS45 is probably involved in vesicle fusion at the TGN and not in transport from the TGN to the PVC, as suggested for yeast Vps45p. However, we cannot exclude the possibility of AtVPS45 being present transiently at the PVC with the bulk pool of the protein residing at the TGN. In yeast, Vps45p and Tlg2p have been shown to function in the Cvt pathway, a pathway that also requires Vti1p, in the generation of transport intermediates (Abeliovich et al., 1999). Intriguingly, because AtVTI1b is able to replace yeast Vti1p in the Cvt pathway, a pathway that also requires Vti1p, in the generation of transport intermediates (Abeliovich et al., 1999). Thus, the presence of Vti1p (or AtVTI1a) in Arabidopsis root extracts may suggest that AtVPS45, AtTLG2a or -b, and AtVTI1b may function together in a similar Cvt pathway in Arabidopsis. However, it is not clear that such a transport pathway exists in plant cells, although this remains an exciting possibility for further investigation.

Several other models can also be envisaged for the function of AtVPS45. One possibility is that it may be involved in vesicular transport from the PVC back to the TGN. This transport pathway would be required for the recycling of membrane lipids and proteins of the transport machinery, such as v-SNAREs and cargo receptors, for additional rounds of transport to occur. The colocalization of the vacuolar cargo receptor AtELP with AtVPS45 at the TGN may indicate sites of fusion of recycling vesicles containing AtELP with the TGN. A second possibility is that AtVPS45 plays a role in endocytosis from the plasma membrane. Although little is known about this process in plant cells, it has been studied extensively in other organisms, and at least two pathways for endocytosis from the plasma membrane to the TGN are apparent in mammalian cells (Mallet and Maxfield, 1999). One endocytic pathway may pass through the PVC and converge with the recycling pathway at this point, but other pathways may involve distinct populations of
endosomes and different transport machinery. In yeast, Tlg2p has been implicated in endocytosis, in particular in the recycling of the plasma membrane v-SNARE Snclp (Lewis et al., 2000), and AtTlg2a or -b may function along with AtVPS45 in this process. Alternatively, AtVPS45 and AtTLG2a or -b may be required for anterograde transport through the Golgi apparatus to the TGN. It is not known in Arabidopsis whether protein transport through the Golgi occurs by vesicle transport or cisternal maturation (Bonfanti et al., 1998) or a combination of the two; however, AtVPS45 could be involved in either of these processes.

To distinguish between these different possibilities, Arabidopsis mutants lacking AtVPS45, AtTLG2a, or AtTLG2b are being isolated. However, although we can isolate potential knockout insertions as heterozygotes, homozygous knockout mutants have yet to be recovered, indicating that these may be essential genes. Therefore, we are attempting to generate conditional mutants to assess function directly; however, this is an area that is not well developed in plants. If we are able to isolate these conditional mutants, a detailed study of the mutant phenotypes with respect to their transport of various vacuolar and secreted proteins along the secretory pathway, the morphology of endomembrane compartments, and the localization of other components of the vesicle transport machinery should provide information regarding the vesicle fusion step these proteins are involved in. Because plant cells contain a highly complex endomembrane system that changes with specific cell type and developmental stage (Jauh et al., 1999), the roles of AtVPS45, AtTLG2a, and AtTLG2b and the transport pathways they function in will need to be examined throughout development and in many different plant tissues. The effect of the loss of these proteins, and of the corresponding vesicle-trafficking pathway, on the growth and development of the plant should yield insight into the importance of the pathway to the whole organism.

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