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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.

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-SNAREs function 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 PVC, 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 interacts with the TGN or endosomal t-SNARE Tlg2p, and Tlg2p appears to provide 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 Snc1p 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 coimmunoprecipitates 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], AtVAM3 [Sato *et al.*, 1997], AtPLP [Zheng *et al.*, 1999a], KNOLLE [Lukowitz *et al.*, 1996], and AtSYR1 [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. Because

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 referred 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′ to the predicted ORF (TLG2b-F1: GCT CCG ATT TTG TTT ATT TTC TCC; TLG2b-R1: GGC CAA GAG AGG GTT ACT GTT 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 *Xba*I and *Nde*I restriction sites directly upstream of the start codon and an *Xho*I site immediately after the stop codon (TLG2a-F: GG TCT AGA CAT ATG GCG ACG AGG AA; TLG2a-R: GG CTC GAG TCA CAA GAA TAT TTC CT; TLG2b-F2: GG TCT AGA CAT ATG GCG ACG AGG AA; TLG2b-R2: GG CTC GAG GAC TAA AAC 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.

A third novel sequence, corresponding to a predicted gene (MJJ3.6) from a 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 *Hin*dIII and *Nde*I sites at the 5′ end and an *Xho*I site at the 3′ end of the ORF (SED5-F: GG AAG CTT CAT ATG GCG ACG AGG AA; SED5-R: CC CTC GAG CTA AGC CAC AAA GAA GAA GG). This amplified product was cloned into pBluescript KS (Stratagene) to create pNde-SED5.

All of these cDNAs were completely sequenced with the use of dye-primer dideoxy sequencing at the Michigan State University Sequencing Center. The sequence of each t-SNARE has been deposited into GenBank under accession numbers AF067789 (AtTLG2a), AF154574 (AtTLG2b), and AF051853 (AtSED5). The cDNAs were predicted to encode typical syntaxin-type t-SNAREs of 322 (AtTLG2a), 323 (AtTLG2b), and 336 (AtSED5) amino acids.

Yeast Expression of Arabidopsis t-SNAREs

The plasmid pH6-Nde is a derivative of pBluescript KS in which the sequence between the *Bam*HI and *Pst*I restriction sites has been replaced by a linker encoding a start codon followed by seven histidine residues and an *Nde*I restriction site (GATCA ATG CAT CAT CAT CAT CAT CAT CAT ATG CTGCA). The *Nde*I–*Xho*I 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 *Nde*I–*Hin*dIII fragment of pNde-PEP12 (Sanderfoot *et al.*, 1999) was cloned into similarly digested pH6-Nde to create pH6-AtPEP12. The *Bam*HI–*Eco*RI fragment of a plasmid containing the *AtVAM3* cDNA, which was engineered to contain a *Bam*HI restriction site immediately upstream of the start codon (Sanderfoot *et al.*, 1999), was cloned into pET-28a, creating pH6-AtVAM3. The DNA encoding these 6x-His–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 the 6x-His tag and with specific antisera to each t-SNARE, where available.

For expression of untagged AtTLG2a and AtTLG2b in yeast cells, the *Xba*I–*Xho*I 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 (Schena 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 6x-His 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 KPO_4, 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 *Xho*I restriction site immediately before the first codon of the transmembrane anchor (TLG2a-RΔ: TCA CTC GAG CTT CAC CAT ACC TCC A). This amplified product (which encodes for amino acids 1–299 of AtTLG2a) was cloned into *Xba*I–*Xho*I-digested pBluescript, creating pNde-TLG2aΔTM.

For bacterial expression, the *Nde*I–*Xho*I 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 (Reamstown, 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 amylose resin. A translational fusion of amino acids 1–299 of AtTLG2a to the C terminus of maltose-binding protein was created by cloning the *Xba*I–*Xho*I fragment of pNde-TLG2aΔTM into *Xba*I–*Sal*I-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 amylose affinity columns as described by the manufacturer (New England Biolabs). The amylose columns containing bound protein were equilibrated with 100 mM sodium borate, pH 9.0, before dimethyl pimelimidate was added to a final concentration of 4 mg/ml. After incubation at room temperature for 30 min, excess cross-linker was quenched 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 *Bam*HI restriction site at the start of the ORF and a *Sal*I restriction site just before the transmembrane domain (VTI1b-F: AAG GAT CCA GAT GAG CGA CGT ATT TGA A, VTI1b-R: AAG TCG ACA TTT GTT CCT AGT CAT TCT). The PCR fragment was cloned into *Bam*HI–*Sal*I-digested pET-14b (Novagen). Recombinant His-tagged AtVTI1b was overexpressed and purified with the use of a Ni^{2+} 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 *Nde*I and *Xho*I 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 *Bgl*II and *Xho*I 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 *Xba*I, 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 *Xho*I. This DNA fragment was cloned into pET-23b (Novagen) that was digested with *Bam*HI, 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 *Xho*I, 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 5′ primer that coded for the nine-amino acid HA tag (AAG GAT CCA TGT ACC CGT ACG ATG TGC CGG ATT ACG CTA GCG ACG TAT TTG AAG G) together with a 3′ primer specific for *AtVTI1b* (CAA GTA ACC ACT TGG GCC AT). The *HA-AtVTI1b* construct was confirmed by sequencing and was able to complement the yeast *vti1* mutant in the same way as untagged *AtVTI1b* (Zheng *et al.*, 1999b; H. Zheng, G. Fischer von Mollard, N.V. Raikhel, unpublished). *HA-AtVTI1b* was cloned behind the constitutive cauliflower mosaic virus 35S promoter and introduced into *Arabidopsis* as described above.

Immunoprecipitation of Detergent Extracts of Arabidopsis Roots

AtVPS45 antibodies were affinity purified against *E. coli*–synthesized AtVPS45 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 $1000 \times g$, and the supernatant was further centrifuged at $100{,}000 \times g$ 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 repelleted at $100{,}000 \times g$, 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 nonstoichiometrically 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, 3% [wt/vol] SDS, 3% [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,

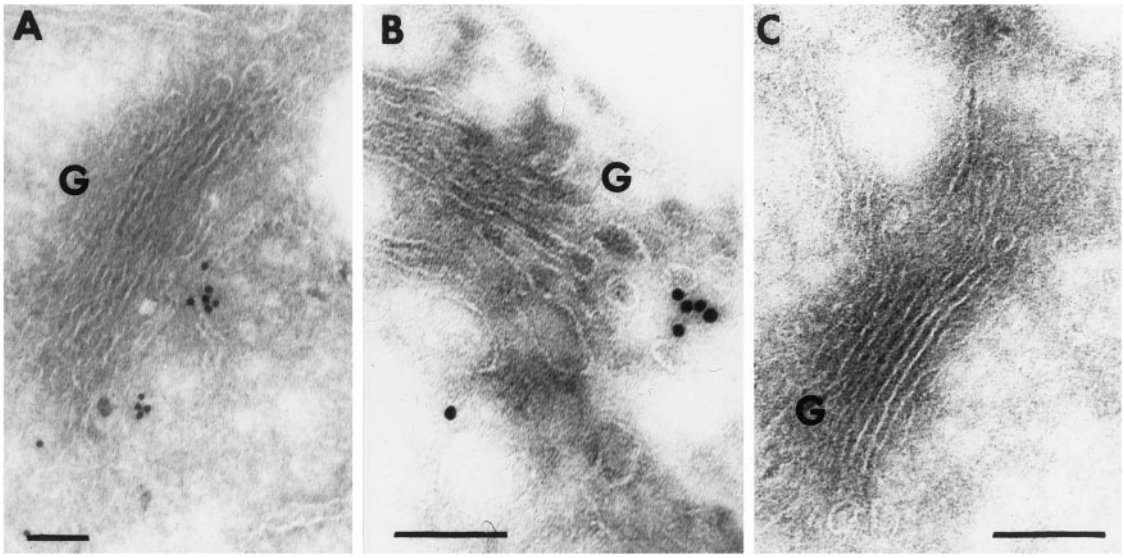

Figure 1. Immunolocalization of AtVPS45 on ultrathin cryosections from *Arabidopsis* roots. (A and B) Sections were incubated with AtVPS45 antibodies followed by biotinylated goat anti-rabbit secondary antibodies and detected with the use of streptavidin conjugated to 10-nm gold particles. (C) Control section probed as for A and B except for the use of preimmune serum in place of the AtVPS45 antibodies. G, Golgi. Bar, 0.1 μm.

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 AtVAM3 (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). AtTLG2a 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

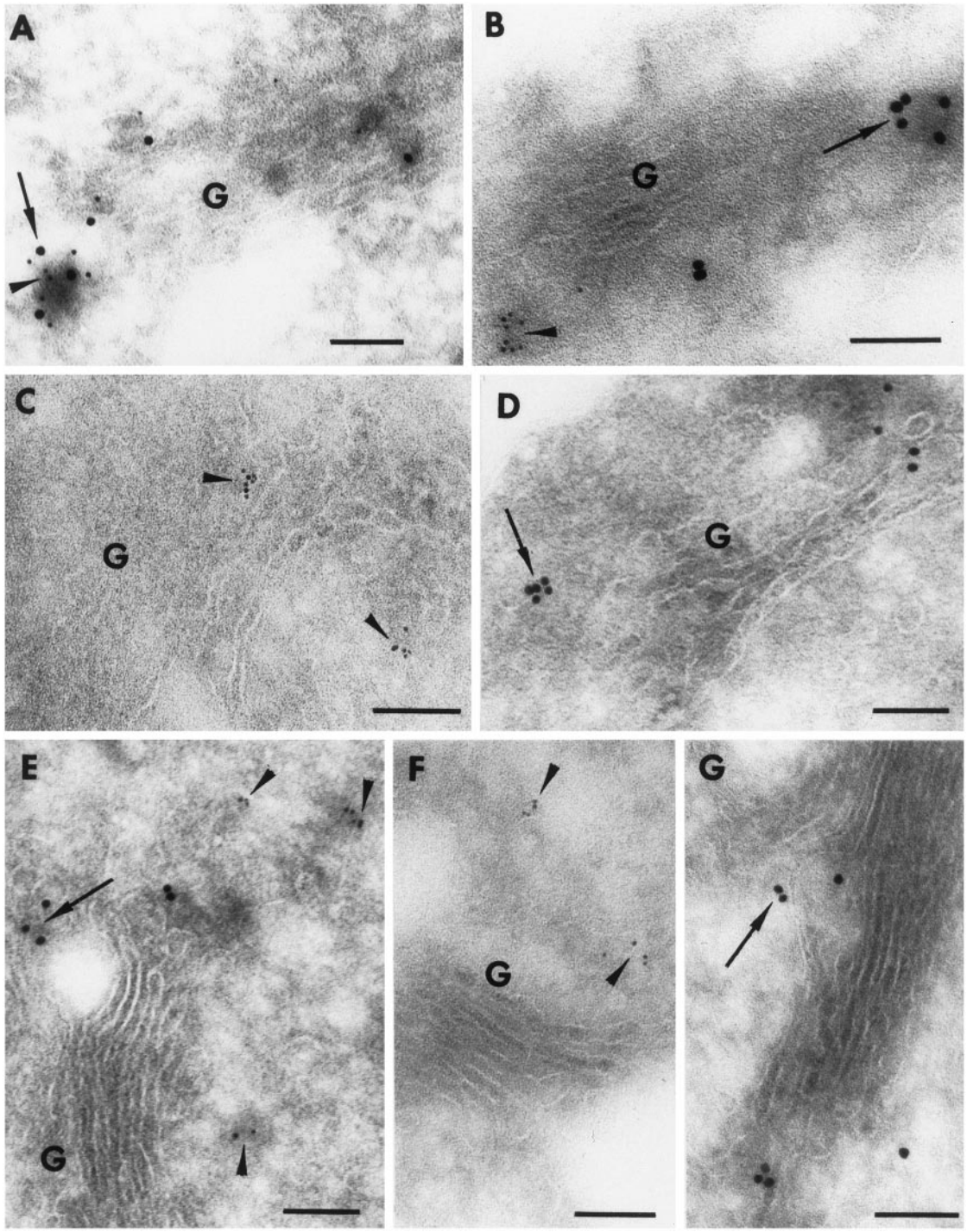

Figure 2. AtVPS45 colocalizes with AtELP, and not with AtPEP12, on ultrathin cryosections of *Arabidopsis* roots. (A and B) Double labeling for AtELP and AtVPS45. Sections were incubated with AtELP antibodies followed by biotinylated goat anti-rabbit secondary antibodies and detected with the use of 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.

Table 1. Quantitation of immunogold electron microscopy double labeling

	Separate	Together	Total
AtVPS45 + AtELP	23	27	50
AtVPS45 + AtPEP12	28	2	30
AtVPS45 + AtTLG2a	27	27	54
AtVPS45 + AtTLG2b	13	13	26
AtTLG2a + AtTLG2b	22	2	24
AtTLG2a + AtELP	22	9	31
AtTLG2b + AtELP	3	16	19
AtVPS45 + AtVTI1a	11	21	32
AtVPS45 + AtVTI1b	23	10	33

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.

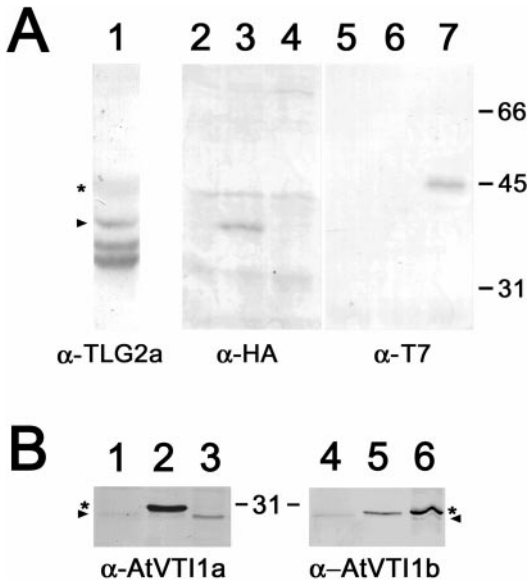

Figure 4. Characterization of antibodies and epitope tags for AtTLG2a/b and AtVTI1a/b. (A) A microsomal extract of wild-type *Arabidopsis* (lane 1) was probed with affinity-purified antiserum to AtTLG2a. Full-length AtTLG2a is indicated by the arrowhead, and the smaller bands represent proteolytic breakdown 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.

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 -b (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-

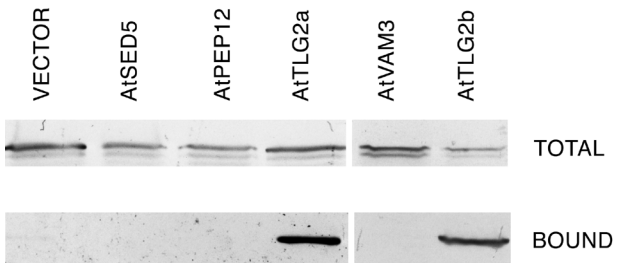

Figure 3. AtVPS45 interacts with AtTLG2a and AtTLG2b in a yeast expression system. Yeast were cotransformed with cDNAs encoding the His-tagged t-SNAREs AtSED5, AtPEP12, AtTLG2a, AtVAM3, or AtTLG2b, or with vector alone as a control, and untagged AtVPS45. The t-SNAREs were isolated from the yeast extracts 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.

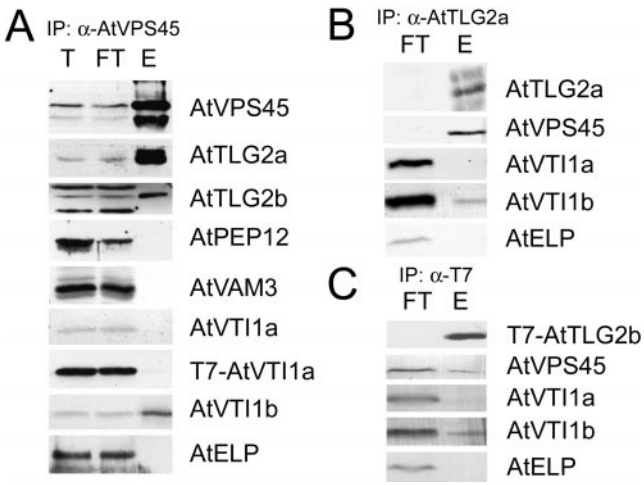

Figure 5. AtTLG2a, AtTLG2b, and AtVTI1b coimmunoprecipitate with the use of AtVPS45 antibodies. (A) Detergent-solubilized membrane preparations from *Arabidopsis* roots grown in liquid culture were subjected to immunoisolation with the use of AtVPS45 antibodies. Aliquots of the total extract (T), the flow through after immunoprecipitation (FT), and the eluate from the antibody column (E) were analyzed by SDS-PAGE and immunoblotting with the indicated antibodies. In the case of T7-AtTLG2b and T7-AtVTI1a, the immunoprecipitations were performed with the use of tissue from transgenic plants expressing the indicated epitope-tagged protein and probed with the use of mAbs against the T7 tag. (B and C) Detergent-solubilized membranes from wild-type (B) or *T7-AtTLG2b*–expressing (C) *Arabidopsis* roots grown in liquid culture were subjected to immunoisolation with AtTLG2a antibodies (B) or T7 mAbs (C). The entire flow through was precipitated by trichloroacetic acid, and equal amounts of flow through and eluate were analyzed by SDS-PAGE and immunoblotting with the indicated antibodies.

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 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-

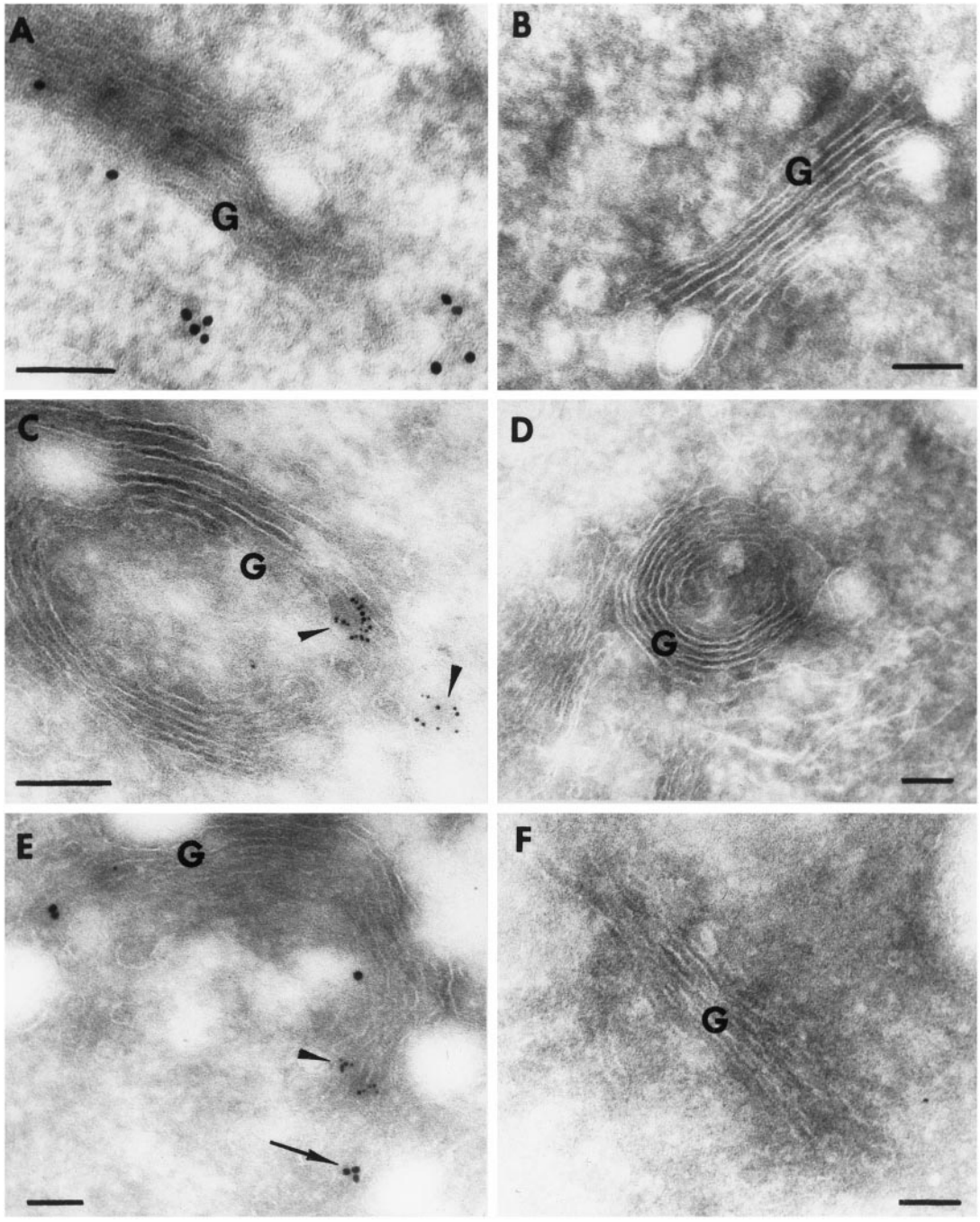

Figure 6. AtTLG2a and AtTLG2b localize to different subdomains of the TGN. (A) Ultrathin cryosections of *Arabidopsis* roots were labeled with AtTLG2a antibodies followed by biotinylated goat anti-rabbit secondary antibodies and detected with the use of streptavidin conjugated to 10-nm gold particles. (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.

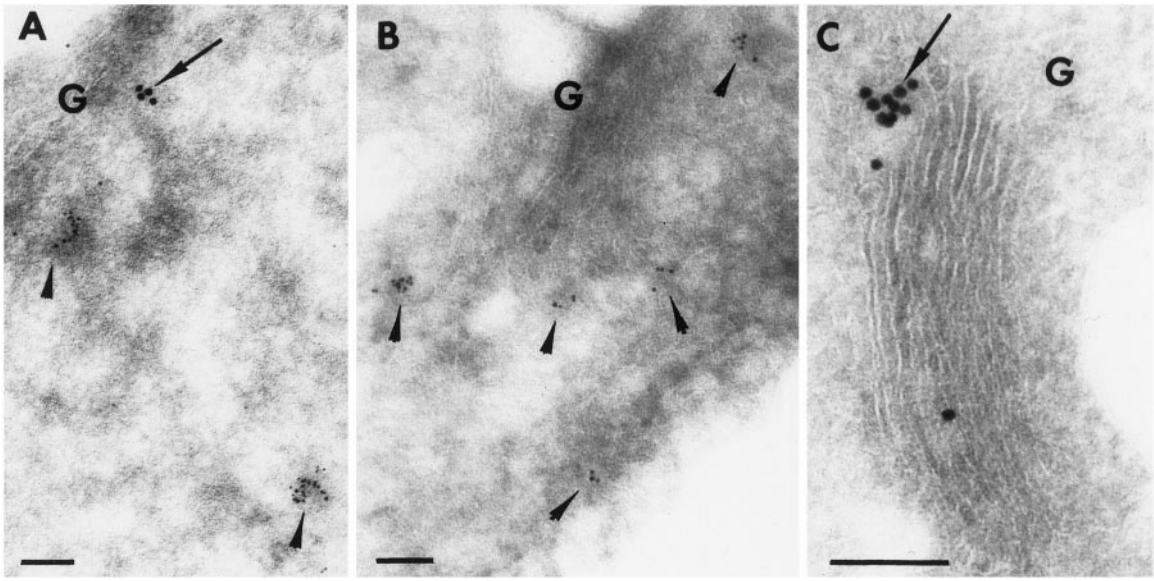

Figure 7. AtTLG2a is not present on the PVC. (A) Double labeling was performed on cryosections from *Arabidopsis* roots as described for Figure 2 with the use of antibodies against AtPEP12 (5 nm gold) and AtTLG2a (10 nm gold). (B) Control labeled as for A except for the use of AtTLG2a preimmune serum in place of anti-AtTLG2a. (C) Control labeled as for A except for the use of AtPEP12 preimmune serum in place of anti-AtPEP12. G, Golgi. 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 the appropriate controls (Figure 7, B and C). No colocalization could be observed for these two proteins, ruling out the possibility that AtTLG2a is also found at the PVC.

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). 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).

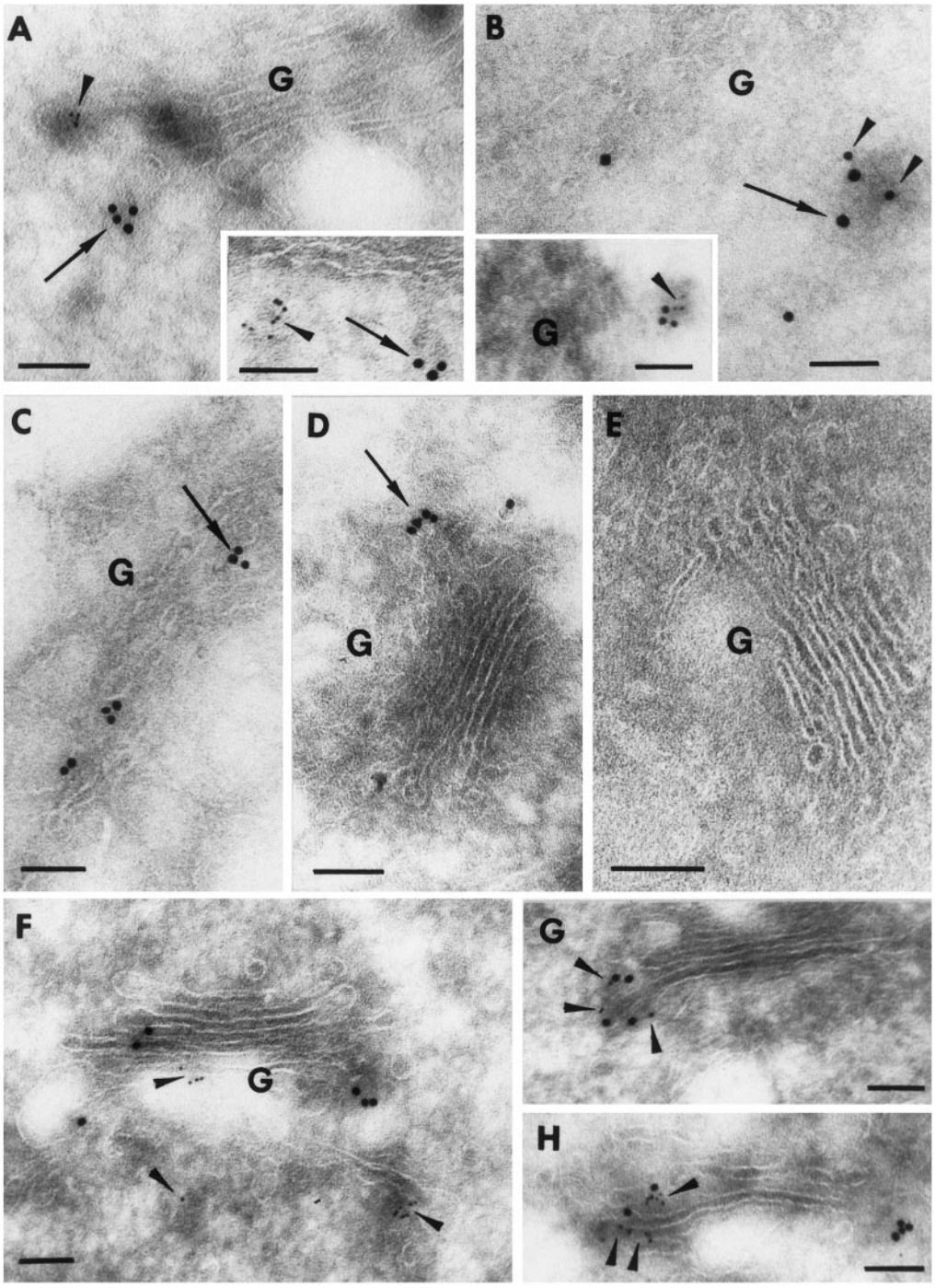

Figure 8. AtTLG2a and AtTLG2b colocalize with AtVPS45. Double labeling was performed as described in Figure 2. (A and inset) AtTLG2a and AtVPS45 on separate parts of the TGN. Cryosections were labeled with AtTLG2a antibodies (5 nm gold) followed by AtVPS45 antibodies (10 nm gold). (B) AtTLG2a and AtVPS45 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.

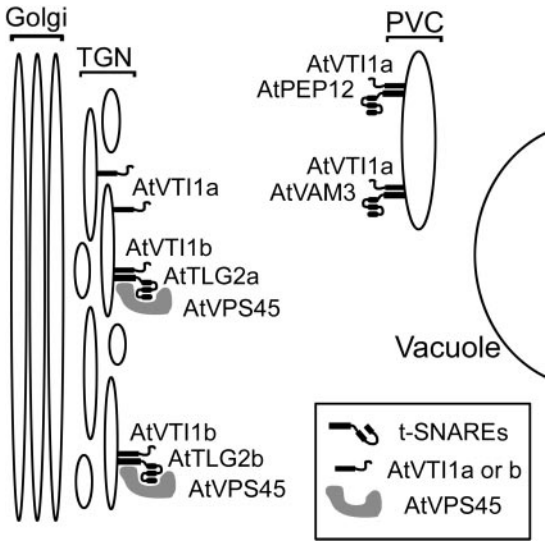

Figure 9. Summary of results. The t-SNAREs AtTLG2a and AtTLG2b reside on distinct domains of the TGN, where each is found in separate complexes with the Sec1p family member AtVPS45. Unlike the homologous yeast Sec1p family member Vps45p, AtVPS45 is not found on the PVC, nor does it interact with the PVC t-SNAREs AtPEP12 and AtVAM3. Although the highly homologous SNAREs AtVTI1a and AtVTI1b are each found on the TGN, only AtVTI1b is found in AtTLG2a-AtVPS45 and AtTLG2b-AtVPS45 complexes. AtVTI1a has been shown previously to be localized also to the PVC and to be found in complexes with the PVC t-SNAREs AtPEP12 and AtVAM3 (Sanderfoot *et al.*, 1999).

AtVTI1a is known to partially localize to the TGN (Zheng *et al.*, 1999b), and immunogold labeling indicated that AtVTI1b is also found on this organelle. Double labeling was performed with the use of transgenic plants expressing T7-AtVTI1a or HA-AtVTI1b and antibodies against AtVPS45 and the epitope tag (Table 1). Both AtVTI1a and -b colocalized in part with AtVPS45 at the TGN.

Based on these data, we propose that AtVPS45 interacts with both AtTLG2a and AtTLG2b, but in different domains of the TGN, potentially functioning in different vesicle fusion reactions. These reactions probably also involve AtVTI1b, although further work is required to elucidate the precise role of each component (Figure 9).

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 AtVAM3 (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: AtVAM3, 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 vesicle 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, the preferential interaction of AtVPS45 with AtVTI1b (rather than AtVTI1a) in *Arabidopsis* root extracts may suggest that AtVPS45, AtTLG2a (or AtTLG2b), 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 Snc1p (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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