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The Syntaxin Homolog AtPEP12p Resides on a Late Post–Golgi Compartment in Plants

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Soluble proteins are transported to the plant vacuole through the secretory pathway via membrane-bound vesicles. Targeting of vesicles to appropriate organelles requires several membrane-bound and soluble factors that have been characterized in yeast and mammalian systems. For example, the yeast PEP12 protein is a syntaxin homolog that is involved in protein transport to the yeast vacuole. Previously, we isolated an *Arabidopsis thaliana* homolog of PEP12 by functional complementation of the yeast *pep12* mutant. Antibodies raised against the cytoplasmic portion of AtPEP12 have been prepared and used for intracellular localization of this protein. Biochemical analysis indicates that AtPEP12 does not localize to the endoplasmic reticulum, Golgi apparatus, plasma membrane, or tonoplast in Arabidopsis plants; furthermore, based on biochemical and electron microscopy immunogold labeling analyses, AtPEP12 is likely to be localized to a post–Golgi compartment in the vacuolar pathway.

INTRODUCTION

Families of proteins involved in targeting and fusion of vesicles with their acceptor membranes have been identified in various organisms, particularly in mammalian neuronal cells and in yeast. These studies have led to the proposal of a general model for the docking of vesicles with their target membranes (SNARE or SNAP receptor hypothesis). According to this hypothesis, a specific v-SNARE (e.g., synaptobrevin) in the vesicle membrane interacts with a specific t-SNARE (e.g., syntaxin and SNAP-25) in the target membrane, along with the soluble factors α-SNAP and NSF, which seem to be common components found at each stage of the secretory pathway. These t-SNARES and v-SNARES enable the vesicle to dock at the target membrane and permit fusion to occur (Bennett et al., 1992a, 1992b; Söllner et al., 1993a, 1993b; Calakos et al., 1994). Different isoforms of t-SNAREs and v-SNAREs reside in various cell membranes and vesicles, possibly providing specificity for the docking reaction.

We previously isolated an *Arabidopsis thaliana* cDNA (*AtPEP12*) by using functional complementation of the yeast *pep12* mutant and found it to be homologous to the yeast *PEP12* gene and other members of the syntaxin family (Bassham et al., 1995). The yeast PEP12p most likely functions in the transport of vacuolar hydrolases and is thought to be associated with an endosome-like compartment (Becherer et al., 1996), suggesting a similar role for AtPEP12p. The C-terminal portion of all syntaxins is highly conserved (Aalto et al., 1993; Bennett et al., 1993), and this region of the AtPEP12p also displays the highest homology to other syntaxins, including the KNOLLE protein, which is the only other available syntaxin from plants (Lukowitz et al., 1996). The extreme C terminus of AtPEP12p is highly hydrophobic and is predicted to form a membrane-anchoring domain. Most syntaxins are transmembrane anchored at their C terminus, with the bulk of the protein facing the cytosol. This orientation is consistent with the proposed function for syntaxins as transport vesicle receptors facilitating interaction with components on the vesicular membrane (Bennett, 1995; Rothman and Wieland, 1996). In this report, we describe our continuing studies designed to localize AtPEP12p in plant cells. Our overall goal is to address the precise function of this protein in plants.

RESULTS

Tissue Distribution and Properties of AtPEP12p

Hydropathy plot analysis predicts that AtPEP12p, similar to other members of the syntaxin family, possesses a transmembrane anchor domain at the C terminus; therefore,

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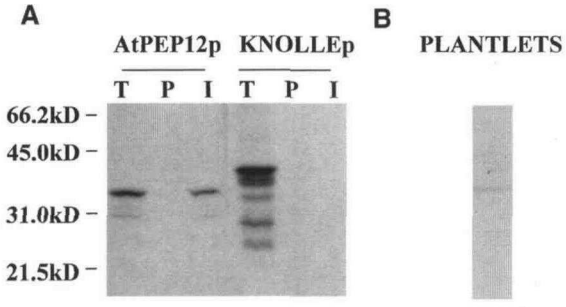

Figure 1. AtPEP12p Antibodies Specifically Immunoprecipitate the AtPEP12 Protein.

(A) *AtPEP12* and *KNOLLE* products were translated in vitro (T) and immunoprecipitated with either AtPEP12p immune (I) or preimmune (P) sera.

(B) AtPEP12p antibodies were used against protein crude extracts from Arabidopsis plantlets.

The positions of molecular mass markers are indicated at left.

polyclonal antibodies were raised against a presumed cytosolic portion of AtPEP12p. Syntaxins belong to a family of related proteins; thus, to characterize the specificity of our antibodies, we used KNOLLE, the only other available plant protein belonging to the syntaxin family. The KNOLLE protein is comprised of 310 amino acids (significantly longer than the 279 amino acids of AtPEP12p); nevertheless, it possesses all structural features characteristic of syntaxins. The immunoprecipitable products of the in vitro–translated *AtPEP12* and *KNOLLE* genes were compared. The major in vitro translation product of the *AtPEP12* clone was ~35 kD, which is larger than the 31 kD predicted from the deduced sequence of AtPEP12p. This protein was immunoprecipitated by our AtPEP12p antibodies; however, no cross-reactivity was observed with a preimmune serum (Figure 1A). In vitro translation of the KNOLLE protein yielded a major product of 41 kD; in addition, many other proteins of smaller size, possibly derived from KNOLLE, were detected (Figure 1A). However, neither the major nor any of the minor proteins were immunoprecipitated by our AtPEP12p antibodies (Figure 1A), suggesting that our serum specifically recognizes AtPEP12p. These antibodies recognized a major protein of ~36 kD in total cell extracts from seedlings on immunoblots (Figure 1B), and no cross-reactivity with the preimmune serum was observed (data not shown).

AtPEP12p is predicted to be a membrane protein; therefore, isolation of microsomal membranes from various tissues by differential centrifugation at 100,000*g* (P100) was performed. Immunoblot analysis showed AtPEP12p to be strictly associated with the membrane fractions and not with the supernatant (Figure 2). Furthermore, AtPEP12p was not disassociated from membrane fractions in the presence of high salt (0.5 M NaCl) and was solubilized only in the presence of detergents (data not shown). These results are consistent with the prediction that AtPEP12p is an integral membrane protein.

Analysis of AtPEP12p expression in different tissues demonstrated the level of this protein in leaves to be significantly lower than in roots and other tissues (Figure 2), supporting our previous observation that mRNA levels differ among these tissues (Bassham et al., 1995). Interestingly, either one or a range of two to three bands was recognized by the AtPEP12p antibodies, depending on the tissues analyzed. In leaves and siliques, the antibodies recognized a single major band of 36 kD (Figure 2); in contrast, in roots, cell suspension cultures, and flowers, a doublet of 36.0 and 34.5 kD was observed (Figure 2), with an additional band of 32.5 kD that was apparent in some root and cell suspension membrane preparations (Figure 2). The membrane insertion and identity of the AtPEP12p bands were analyzed further.

AtPEP12p Is Anchored at the C Terminus in Membranes and Has Two Major Isoforms

To determine whether AtPEP12p is a type II membrane protein (anchored at the C terminus) as predicted based on the SNARE hypothesis (Bennett, 1995; Rothman and Wieland, 1996), the membrane orientation of this syntaxin homolog was investigated in cell suspension cultures and root extracts. The analysis was based on the sensitivity of the AtPEP12p to various proteases in vesicle extracts that had been osmotically stabilized to maintain their lumenal contents. When vesicle extracts from Arabidopsis roots were treated with the acid protease papain, AtPEP12p was found to be effectively degraded in both the presence and absence of 1% Triton X-100 (Figure 3A). In these same extracts, vacuolar phytohemagglutinin (von Schaewen et al., 1993) was found to be protected from degradation by papain in the absence of detergent (Figure 3), indicating that the vesicle membranes were still intact. Similarly, in extracts from cell suspensions, AtPEP12p was efficiently degraded by papain in the absence of detergent with a half-time of ~15 min (Figure 3B), whereas the endoplasmic reticulum

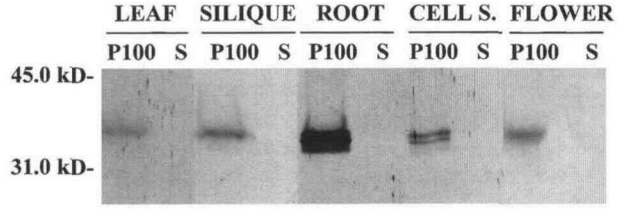

Figure 2. AtPEP12p Is Membrane Associated.

Expression of the AtPEP12 protein was analyzed in several Arabidopsis tissues (leaves, siliques, roots, cell suspension cultures [CELL S.], and flowers). Microsomal membrane pellets (P100) and cytosol (S) were separated by centrifugation at 100,000*g*. Equal amounts of protein were loaded in each lane. The positions of molecular mass markers are indicated at left.

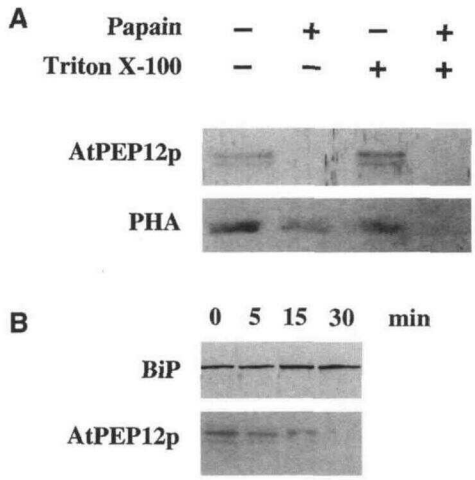

Figure 3. AtPEP12p Is a Type II Membrane Protein.

(A) Membrane vesicles were extracted from phytohemagglutinin-expressing Arabidopsis roots. The samples were digested with papain (+) or left untreated (−) in either the presence (+) or absence (−) of 1% Triton X-100. Proteins were separated by SDS-PAGE and probed for the presence of AtPEP12p or phytohemagglutinin (PHA) by using specific antisera.

(B) Membrane vesicles were extracted from Arabidopsis cell suspensions. The samples were digested with papain in the absence of detergent. Aliquots were removed, and the protease was inactivated at the indicated times. Proteins were separated by SDS-PAGE and probed for the presence of AtPEP12p or lumenal binding protein (BiP) by using specific antisera.

(ER) lumenal binding protein, BiP (Pedrazzini and Vitale, 1995; Bar-Peled and Raikhel, 1997), was completely protected from degradation (Figure 3B). These results showed that AtPEP12p is a membrane protein, with most of the protein being oriented toward the cytoplasm. This result is consistent with the role of AtPEP12p as a t-SNARE in protein trafficking as well as with what has been observed for other members of the syntaxin family (Bennett et al., 1992a, 1993; Becherer et al., 1996).

The nature of the AtPEP12p double band was analyzed to ascertain whether it was the result of proteolysis. The addition of several protease inhibitors (EDTA, phenylmethylsulfonyl fluoride [PMSF], leupeptin, and chymostatin) to the membrane isolation protocol did not result in a single band. Furthermore, AtPEP12p was found to be resistant to degradation by several common proteases (protease K, trypsin, and pepsin) in the absence of detergent, and even in the presence of 1% Triton X-100, protease-resistant fragments were found for trypsin and pepsin (data not shown). These observations indicate that the AtPEP12p doublet was not a result of protein degradation. Other experiments addressing the nature of these doublets were performed with transgenic Arabidopsis plants. Could alternative splicing or post-translational modification have been the cause of these two bands?

Accordingly, we overexpressed AtPEP12p in transgenic Arabidopsis plants by introducing the *AtPEP12* cDNA under the control of a caulifower mosiac virus 35S promoter. Several independent transformants were generated and screened by using crude extracts of plantlets. To analyze further the transgenic lines overexpressing AtPEP12p, membrane and soluble fractions of roots were isolated. The analyses of homozygous and heterozygous transgenic lines compared with wild-type plants showed increased intensity in both bands in the AtPEP12p doublet (Figure 4). These results, in combination with the observation of only a single band on RNA gel blots (Bassham et al., 1995), indicate that the two bands observed are unlikely to be the result of alternative splicing of the *AtPEP12* gene. Our overexpression analyses support the hypothesis that AtPEP12p is post-translationally modified; however, they do not preclude the alternative explanation that a second protein, which is different from AtPEP12p but cross-reactive with AtPEP12p antibodies, is upregulated in transgenic plants.

Biochemical Subcellular Localization of AtPEP12p

A major goal of this work was to localize AtPEP12p to a particular compartment. To investigate the intracellular location of AtPEP12p in roots and cell suspension cultures, we employed subcellular fractionation of Arabidopsis tissues expressing the two major isoforms of AtPEP12p in the greatest relative amounts. Step sucrose gradients from roots were used to determine whether AtPEP12p cofractionates with several available markers for membrane compartments. First, we investigated whether AtPEP12p was associated with the ER. The Arabidopsis SEC12 protein (AtSEC12p) was used as the ER membrane marker (Bar-Peled and Raikhel, 1997). This analysis showed that in the presence of EDTA, AtSEC12p

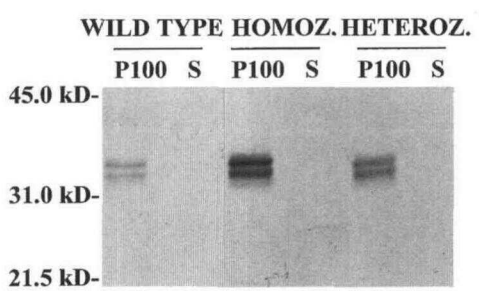

Figure 4. AtPEP12p Has Two Major Isoforms.

Roots from transgenic Arabidopsis plants overexpressing AtPEP12p were fractionated as described in the legend to Figure 2. The increased expression of AtPEP12p was analyzed in homozygous (HOMOZ.) and heterozygous (HETEROZ.) lines for single insertion transgenes. Equal amounts of protein were loaded for the P100 fractions and separately for the S100 fractions. The positions of molecular mass markers are indicated at left.

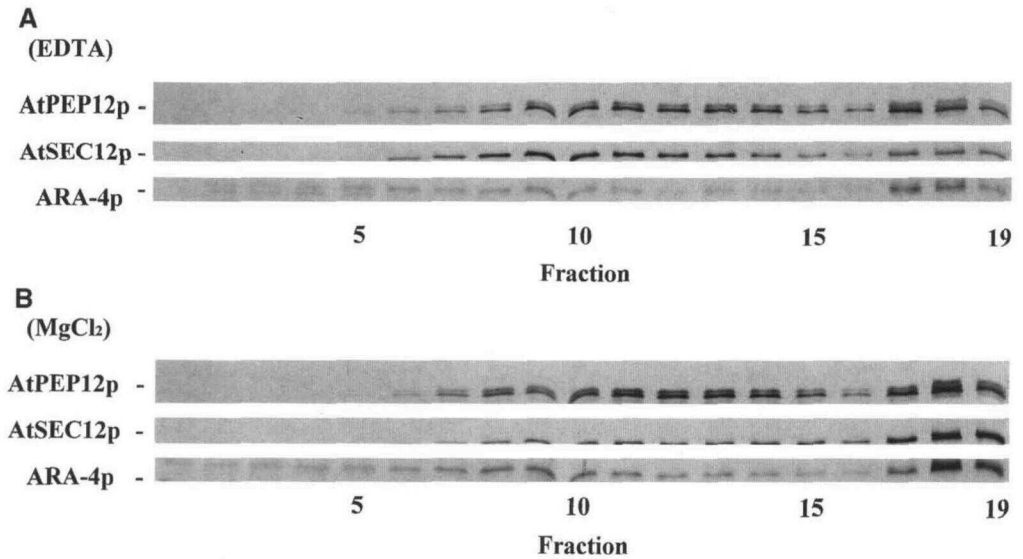

Figure 5. AtPEP12p Does Not Fractionate as Either an ER or a Golgi Membrane Protein.

Sucrose step gradients were used to fractionate microsomal membranes extracted from Arabidopsis roots. Equal volume fractions (one-tenth) were analyzed by SDS-PAGE. The fractionation of AtPEP12p was compared with the distribution of available membrane markers for ER (AtSEC12p) and Golgi (ARA-4p).

(A) Fractions separated in the presence of EDTA.

(B) Fractions separated in the presence of $MgCl_2$.

and AtPEP12p were coenriched between fractions 9 and 14; the 26.5 to 33.5% sucrose interphase corresponds to fractions 9 and 10. AtPEP12p (and AtSEC12p) has a second peak at fraction 18, corresponding to the 40.0 to 56.0% sucrose interphase (Figures 5A and 5B). However, whereas AtPEP12p migrated identically in the presence of either EDTA or Mg^{2+}, AtSEC12p shifted toward the bottom of the gradient, that is, high sucrose concentrations, in buffer containing 5 mM $MgCl_2$ (Figure 5B). This behavior of AtSEC12p is characteristic of ER proteins (Lord, 1987).

The same gradients were used to analyze the distribution of AtPEP12p compared with ARA-4p, which was employed as a Golgi marker (Ueda et al., 1996). Transgenic Arabidopsis plants overexpressing ARA-4p under the control of a heat shock–inducible promoter (Ueda et al., 1996) were used. This analysis showed that ARA-4p has a sharper peak at fraction 9 than does AtPEP12p. Furthermore, a significant amount of ARA-4p remained at the top of the gradient, differing from AtPEP12p and AtSEC12p (Figure 5). The fractionation pattern of a second Golgi marker, latent IDPase activity, was consistent with the ARA-4p results (data not shown), confirming that AtPEP12p is not associated with the Golgi apparatus.

The distribution of AtPEP12p in relation to the plasma membrane and tonoplast was studied in cell suspension cultures (Figures 6 and 7, respectively). The suspension cultures were used because of the difficulty in isolating the quantity of vacuoles necessary for further analysis using roots.

As shown in Figure 6, the plasma membrane was isolated by two-phase partitioning (Kjellbom and Larsson, 1984). After several partitioning steps, the plasma membrane fraction was gradually depleted of other membranes; however, the fraction containing other membranes always remained contaminated with plasma membrane. The results showed that the plasma membrane fraction is enriched in RD28, a known plasma membrane protein (Daniels et al., 1994), and almost completely depleted of AtPEP12p, which therefore does not reside on the plasma membrane (Figure 6).

As a marker for vacuoles, we used a member of the major intrinsic protein (MIP) family from Arabidopsis: the tonoplast-specific protein γ-TIP (Höfte et al., 1991). Protoplasts prepared from the cell suspension cultures were used to isolate vacuoles by flotation in a three-step Ficoll gradient (Figure 7). Most of the AtPEP12p was found in the pellet and the second interphase of the gradient and did not cofractionate with the tonoplast marker, γ-TIP. Thus, AtPEP12p is not localized to the tonoplast in cell suspension cultures (Figure 7).

Immunocytochemical Localization of AtPEP12p

Subcellular fractionations were useful in analyzing the distribution of AtPEP12p, demonstrating that AtPEP12p was not localized to the ER, Golgi apparatus, tonoplast, or plasma membrane. Due to the lack of other endomembrane protein

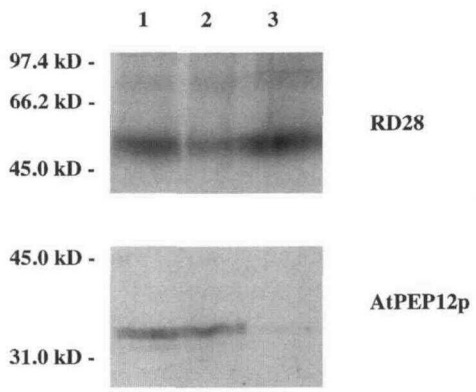

Figure 6. AtPEP12p Does Not Reside on the Plasma Membrane.

Plasma membranes were purified by two-phase partitioning. Lanes 1 contain microsomal membranes; lanes 2, lower phase of the partitioning that contains intracellular membranes; and lanes 3, upper phase enriched for plasma membranes. The protein gel blots were probed with either RD28 or AtPEP12p antibodies. Equal amounts of proteins from the microsomal membranes and lower phase were loaded, compared with three times less of the enriched plasma membrane fraction. The positions of molecular mass markers are indicated at left.

markers in plants, our AtPEP12p compartmentalization analysis could not be extended further using subcellular fractionation approaches. The membrane systems analyzed above are the only ones in the plant secretory pathway for which membrane markers are available. Therefore, electron microscopic immunogold labeling was employed to clarify the identity of the organelle in which AtPEP12p resides. Preliminary attempts to localize AtPEP12p by conventional immunoelectron microscopy in leaves and roots were not successful, probably because of the loss of AtPEP12p antigenicity during fixation. Instead, cryosections were used to immunolocalize AtPEP12p in Arabidopsis roots; we were unable to perform these experiments with other soft tissues.

In root tips, immunoelectron microscopic localization showed that AtPEP12p antibodies labeled small circular ($\leq$0.1 μm in diameter) membrane-bound structures that were scattered throughout the cytoplasm (Figure 8A). The AtPEP12p antibodies did not label the ER, Golgi cisternae, plasma membrane, or tonoplast from mature vacuoles (Figures 8A and 9B), confirming the subcellular fractionation results. In a control experiment, γ-TIP antibodies did not label these structures and instead were found to label extensively the tonoplast of mature vacuoles and other components of the secretory pathway, including the ER, Golgi apparatus, and small vacuolar structures but not the plasma membrane (Figures 8B and 9A). In other plant species, similar results have been previously reported for γ-TIP of soybean cotyledons (Melroy and Herman, 1991) and vacuolar H^+-ATPases of oat roots (Herman et al., 1994). Immunological control specimens treated with preimmune sera were free of label (data not shown). From the thin cryosections, it is almost impossible to assess the three-dimensional extension of the labeled structures and to determine whether they are vesicular or reticulotubular elements. In the most negatively contrasted cryosections, the labeled structures appear electron dense, and the membrane boundary is evident in our thinnest cryosections. The majority of labeling with AtPEP12p antibodies occurred at a high density on the cytoplasmic surface of the membrane (Figure 8A), as was expected for antibodies raised against the cytoplasmic portion of AtPEP12p (Figure 3).

By combining subfractionation and in situ localization techniques, we have concluded that AtPEP12p is not localized to the ER, Golgi, tonoplast, or plasma membrane. Consistent with our yeast complementation results (Bassham et al., 1995) and the labeling that was found occasionally on vesicles at the *trans* side of the Golgi apparatus (Figure 8A), we propose that AtPEP12p is localized to a late post–Golgi compartment on the vacuolar pathway.

DISCUSSION

Protein trafficking to the yeast vacuole has been shown genetically to require the syntaxin homolog PEP12 (Jones, 1976; Becherer et al., 1996). Syntaxins are integral membrane proteins and members of a receptor family involved in vesicular transport through the secretory pathway (Bennett, 1995; Rothman and Wieland, 1996). In this work, the product of the *AtPEP12* gene, an Arabidopsis syntaxin homolog previously identified by functional complementation in yeast

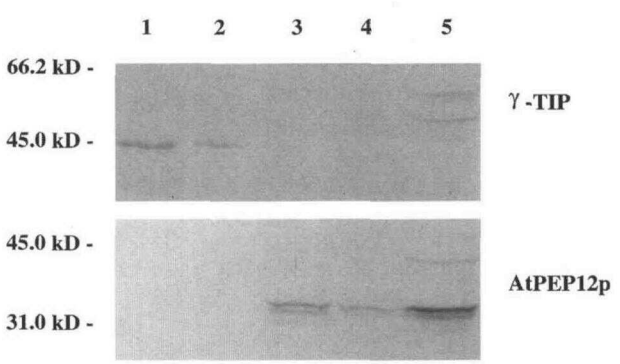

Figure 7. *AtPEP12p Does Not Reside on the Tonoplast.*

Vacuoles were purified by flotation in a three-step Ficoll gradient. Lanes 1 contain floating vacuoles; lanes 2, first interphase; lanes 3, second interphase; lanes 4, bottom layer; and lanes 5, pellet. The protein gel blots were probed with either γ-TIP or AtPEP12p antibodies. Equal amounts of protein from the second interphase, bottom layer, and pellet were loaded, compared with $\sim$10 times less of floating vacuoles and first interphase fractions. The positions of molecular weight markers are indicated at left.

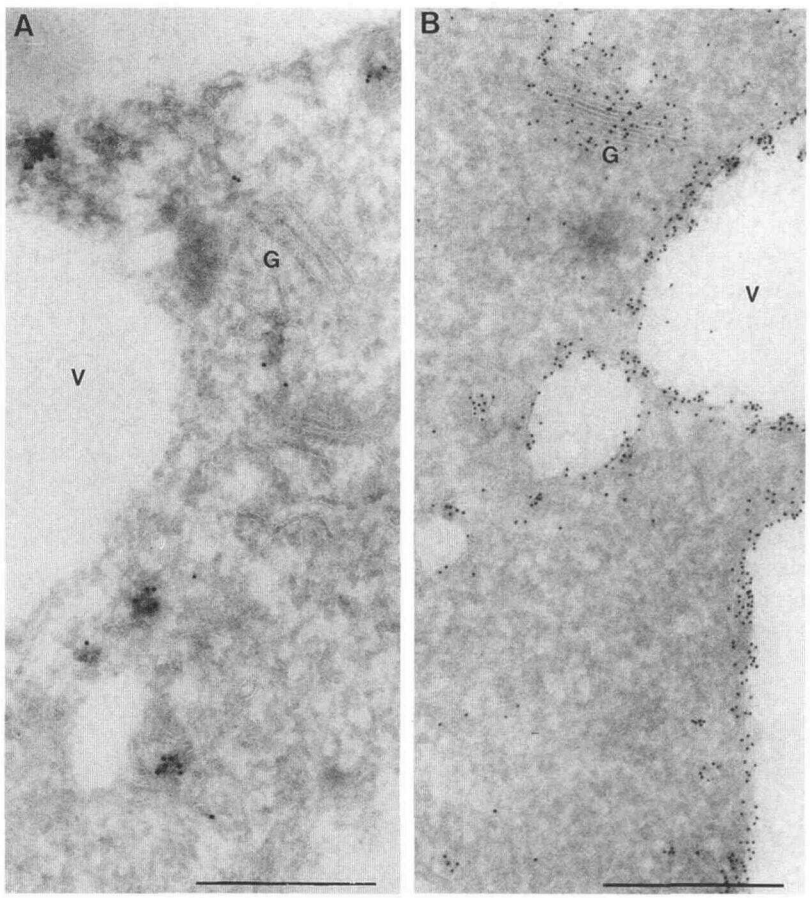

Figure 8. AtPEP12p and γ-TIP Have Different Patterns of Localization.

Cryosections of Arabidopsis roots were immunogold labeled with AtPEP12p and γ-TIP antibodies.
(A) AtPEP12p is localized at the cytoplasmic surface of dense and small (≤0.1 μm in diameter) structures.
(B) γ-TIP is localized to the tonoplast from vacuoles of different sizes and to the Golgi apparatus.
G, Golgi apparatus; V, vacuole. Magnification is ×48,000. Bars = 0.5 μm.

(Bassham et al., 1995), was characterized. Our expression analyses and intracellular localization studies indicate that AtPEP12p is localized to a membrane compartment that may be involved in protein transport to plant vacuoles.

Although it has been suggested that the yeast PEP12p is associated with an endosome-like compartment (Becherer et al., 1996), its exact localization has not yet been determined. We have analyzed the intracellular distribution of AtPEP12p by using subcellular fractionation and immunocytochemical techniques. First, the membrane association and tissue expression of AtPEP12p were addressed. Our analyses show that AtPEP12p is a membrane protein anchored at its C terminus and that it is expressed at relatively low levels in leaves compared with other tissues, which is consistent with our previously observed low levels of mRNA in leaves (Bassham et al., 1995).

Interestingly, two major polypeptides were recognized by the polyclonal antibodies raised against AtPEP12p. The presence of protein doublets corresponding to syntaxin molecules was previously reported, and alternative splicing and post-translational modification have been postulated (Bennett et al., 1993; Ibaraki et al., 1995). We have addressed the former possibility by analyzing AtPEP12p distribution in plants overexpressing the *AtPEP12* gene. Because the level of expression of both polypeptides in transgenic plants was equally increased and only a single band corresponding to *AtPEP12* on RNA gel blots was observed, we consider it unlikely that the bands observed by protein gel blot analysis were the result of alternative splicing. These bands could be explained as the result of post-translational modification. Although AtPEP12p contains a potential N-glycosylation site at residue 83, it is unlikely to be glycosylated because syntaxins have been found to be inserted into membranes post-translationally and thus would be inacces-

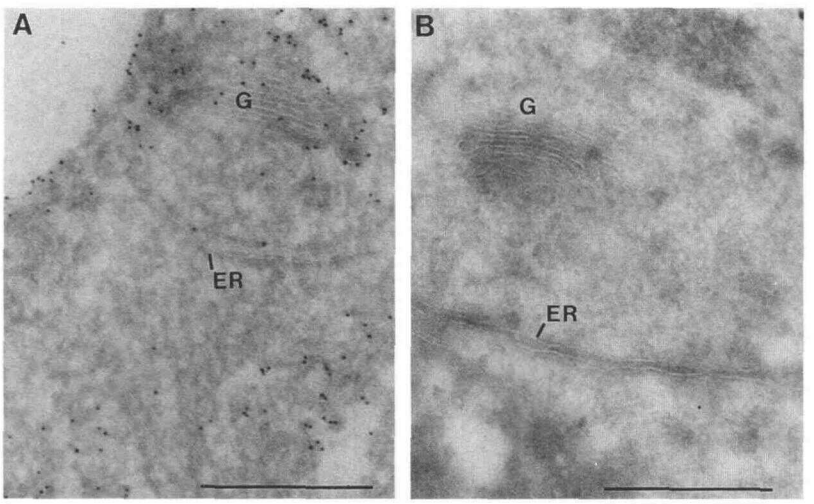

Figure 9. AtPEP12p Does Not Reside on the ER or Golgi Apparatus.

Cryosections of Arabidopsis roots were immunogold labeled with γ-TIP and AtPEP12p antibodies.
(A) The γ-TIP antibodies labeled the tonoplast of the mature vacuole and the membranes of the ER, Golgi apparatus (G), and small vacuoles along the vacuolar biosynthetic pathway.
(B) The same types of organelles are not labeled by the AtPEP12p antibodies.
Magnification is ×47,000. Bars = 0.5 μm.

sible to the N-glycosylation machinery in the ER lumen. Other potential forms of post-translational modification of this protein are currently being investigated.

We analyzed further the localization of AtPEP12p by subcellular fractionation of Arabidopsis roots. Step sucrose gradients obtained from this tissue showed that AtPEP12p is localized to neither the ER nor the Golgi apparatus. Other fractionation techniques demonstrated that AtPEP12p does not reside on vegetative vacuoles or plasma membrane. Cofractionation of the two AtPEP12p isoforms through different procedures further suggested that both polypeptides exist in the same or similar compartments. Due to the difficulties encountered in finding a membrane marker of the plant secretory system that was coenriched with AtPEP12p, the subcellular localization of this syntaxin homolog was established by immunoelectron microscopic analysis of roots. This showed that AtPEP12p is localized to the membranes of a post–Golgi compartment on the vacuolar pathway. The observation that AtPEP12p is not localized to the tonoplast of the mature plant vacuole and yet can complement a yeast vacuolar sorting mutant suggests that there may be another organelle that contains AtPEP12p found between the Golgi apparatus and the vacuole.

Protein transport to the plant vacuole is known to involve different mechanisms. Three classes of vacuolar targeting signals have been identified (Chrispeels and Raikhel, 1992; Gal and Raikhel, 1993; Nakamura and Matsuoka, 1993), and some evidence indicates that transport of soluble proteins to vacuoles is mediated by at least two different pathways (Matsuoka et al., 1995) and two types of transport vesicles (Hohl et al., 1996). Furthermore, in the same plant cell, two functionally distinct vacuole types have been identified (Hoh et al., 1995; Paris et al., 1996). The important question now is whether plants possess one or several types of late post–Golgi compartments on the vacuolar pathway. We must also ascertain whether the vacuolar proteins, using three different targeting signals, are delivered to the main vacuole via the compartment carrying AtPEP12p or whether some unidentified syntaxin proteins are responsible for the delivery of a subset of vacuolar proteins.

One of the predictions of the SNARE hypothesis is that the fusion of transport vesicles with the membrane of an acceptor compartment is specifically determined by the recognition of a v-SNARE molecule by its counterpart t-SNARE (Bennett, 1995; Rothman and Wieland, 1996). On the other hand, it has been shown that rat syntaxins in the plasma membrane are represented by several related proteins (Bennett et al., 1993); similarly, two syntaxin-like proteins in yeast, SSO1p and SSO2p, are localized to the plasma membrane, implying their closely related functions (Aalto et al., 1993). A search for AtPEP12p homologs in the expressed sequence tag data bank showed that syntaxin-like proteins are encoded by several Arabidopsis cDNA clones. By characterizing these clones, we may be able to answer major questions concerning conservation of the machinery described by the SNARE hypothesis among different eukaryotic kingdoms.

Recently, the universality of the SNARE model has been challenged by an increasing pool of data documenting membrane fusion events that do not require NSF or associated factors. One example is vesicular transport from the trans-Golgi

network to apical plasma membrane in Maden-Derby canine kidney cells (Ikonen et al., 1995). In addition, Golgi reassembly in mammalian cells and nuclear envelope fusion in yeast require p97 and CDC48p, respectively. These two proteins share sequence homology with NSF (Acharya et al., 1995; Latterich et al., 1995; Rabouille et al., 1995). However, p97 and CDC48p appear to have a mode of action different from NSF, and they do not require SNAP components (reviewed in Mellman, 1995).

Protein targeting to vacuoles also appears to be a complex variation of the SNARE model. Vesicular transport of proteins from late Golgi and/or a more distal prevacuolar/endosome-like organelle to vacuoles does not require the yeast NSF, SEC18p (Graham and Emr, 1991), although mutations in a t-SNARE homolog (*PEP12*) result in the secretion of Golgi-modified precursors of vacuolar proteins (Becherer et al., 1996). Moreover, the requirement for SEC18p and SEC17p (yeast α-SNAP) in homotypic vacuole fusion occurs during the predocking stage (Haas and Wickner, 1996; Mayer et al., 1996). Whereas all vacuolar protein sorting (*VPS*) genes of the same mutant class as *PEP12* have been cloned (*VPS3*, *VPS6/PEP12*, *VPS9*, *VPS15*, *VPS19/VAC1*, *VPS21*, *VPS34*, and *VPS45*), surprisingly, none of them is homologous to other components of the SNARE complex (Raymond et al., 1990; Herman et al., 1991; Weisman and Wickner, 1992; Schu et al., 1993; Cowles et al., 1994; Horazdovsky et al., 1994; Piper et al., 1994; Becherer et al., 1996; Burd et al., 1996). This leads to the question of how proteins find their way from the trans-Golgi network to the late post–Golgi AtPEP12p organelle and from there to the central vacuole. Despite these observations and the questions raised, ruling out the possibility that the AtPEP12p interacts with other proteins to form a docking/fusion multimeric structure equivalent to SNARE complexes remains premature.

METHODS

Plant Growth Conditions

Arabidopsis thaliana wild-type plants (RLD ecotype) were grown in soil and tissue culture at 22°C with 80% humidity and 14 hr of light. For plants grown in liquid tissue culture to obtain large amounts of roots (Bar-Peled et al., 1995), the medium consisted of 10 g/L sucrose, 4.3 g/L Murashige and Skoog salts (Gibco BRL), 0.5 g/L Mes, 0.1 g/L myoinositol, 1 mg/L thiamine, 0.5 mg/L pyridoxine, and 0.5 mg/L nicotinic acid, pH 5.7. The liquid cultures were maintained under constant agitation (50 to 60 rpm). To select transgenic plants, we used solid medium containing 8 g/L phytoagar and 50 mg/L kanamycin.

Cell Suspension Cultures

The original Arabidopsis cell suspension lines were established by Axelos et al. (1992) and were maintained by weekly subcultures of 10 mL of cell suspension into 60 mL of fresh culture medium (3.2 g/L Gamborg's B5 [Sigma], 20 g/L sucrose, and 2.5 μM 2,4-dichlorophenoxyacetic acid, pH 5.7).

AtPEP12p Expression in *Escherichia coli* and Production of Antibodies

The BglII fragment (621 bp) of *AtPEP12* (GenBank accession number L41651) corresponding to the predicted cytosolic domain was cloned into the BamHI site of pBluescript KS+ (Stratagene, La Jolla, CA) to generate the plasmid pAC3. From pAC3, an EcoRI-NotI fragment containing the *AtPEP12* region was ligated into pGEX-5X-3. The derived plasmid, pAC5, was used to express in *E. coli* the AtPEP12 protein domain as a glutathione *S*-transferase (GST) fusion. The fusion protein was purified over glutathione–Sepharose 4B (Pharmacia), according to the manufacturer's instructions and the method of Bar-Peled and Raikhel (1996). Briefly, the expression of AtPEP12p in *E. coli* liquid cultures (50 mL) growing for 3 hr at 37°C was induced by adding isopropyl β-D-thiogalactopyranoside to a final concentration of 0.2 mM. The cultures were grown at 28°C for an additional 2 or 3 hr, and the cells were collected by centrifugation. The pellet was resuspended in 10 mL of cold extraction buffer (20 mM sodium phosphate, pH 7.0, 0.15 M NaCl, and 1 mM EDTA) containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF). After incubation at 4°C for 5 min, the cells were lysed using a French press at 1100 psi. Triton X-100 was added to a final concentration of 1% (v/v), and the lysate was rocked at 4°C for 30 min and centrifuged at 12,000*g* for 20 min. The supernatant was added to 2 mL of a 50% slurry of glutathione–Sepharose 4B and mixed at room temperature for 20 min. After sedimenting the Sepharose beads (500*g* for 1 min), they were washed four times (5 min each) in extraction buffer containing 0.5% Triton X-100. The AtPEP12p–GST fusion was eluted from the beads in 50 mM Tris-HCl, pH 8.0, and 10 mM reduced glutathione. The eluted protein was mixed with TiterMax adjuvant (CytRx Corporation, Norcross, GA) in a double hub needle, according to the manufacturer's instructions, and injected into rabbits.

AtPEP12 Sense Construct and Plant Transformation

The *AtPEP12* cDNA insert was subcloned into the plant binary expression vector pGA643 and introduced into *Agrobacterium tumefaciens* GV3101(PMP90) by electroporation. Arabidopsis RLD plants were transformed using vacuum infiltration, as described by Bent et al. (1994). Transformants were selected by growth on kanamycin, and the presence of increased amounts of AtPEP12p was detected in several independent lines by protein gel blot analysis.

SDS-PAGE and Immunoblotting

Protein samples were either lyophilized or trichloroacetic acid precipitated before separation by SDS-PAGE, according to Laemmli et al. (1970). The proteins were transferred from gels to nitrocellulose membranes by electroblotting in Tris–glycine buffer at 40 V for 2.5 hr. After blocking (10% nonfat milk powder in PBST), blots were incubated with primary antibodies raised against ARA-4p (1:750), AtPEP12p (1:1000), AtSEC12p (1:500), γ-TIP (1:2000), and RD28 (1:250).

Immunoprecipitation of in Vitro Translation Products

The in vitro transcription mixture (23 μL) of *AtPEP12* or *KNOLLE* contained 1 μg of linearized DNA, 5 μL of 5 × transcription buffer (Promega), 6 μL of rNTPs (ATP, CTP, and UTP; 2.5 mM each), 1 μL of Cap analog (5 mM; Boehringer Mannheim), 1.0 μL of DTT (100 mM), 1.0 μL of RNase inhibitor, and 0.5 μL of RNA polymerase. After 10 min at 37°C, 2 μL of 10 mM GTP was added, and the mixture was incubated for an additional 50 min (37°C). For the in vitro translation reactions in a total volume of 50 μL, 2 μL of RNA was added to 25 μL of wheat germ extract (Promega), 4 μL of amino acid mix without methionine (1 mM of each), and 5 μL of EXPRE ^{35}S protein labeling mix (New England Nuclear–Du Pont). Incubation was done at room temperature for 90 min. Protein A–Sepharose beads were washed several times in TNET 250 buffer (25 mM Tris-HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, and 1% Triton X-100). To 1 to 3 μL of the in vitro translations, 300 μL of TNET 250 and 5 μL of antibodies were added and rocked. After a 90-min incubation, 25 μL of a 50% slurry of protein A–Sepharose was added and rocked for an additional 30 min. The beads were washed three times, 5 min each, with TNET 250. After a final wash in Tris–EDTA buffer (10 mM Tris-HCl, pH 7.5, and 5 mM EDTA), 20 μL of sample buffer was added. The samples were boiled for 3 min and spun in a microcentrifuge, and the supernatant was loaded into SDS–polyacrylamide gels.

Protein Crude Extracts

To prepare crude extracts of proteins (modified from Bar-Peled et al., 1995), we ground fresh tissue in liquid nitrogen and added 4.5 mL of homogenizing buffer (100 mM Tris-HCl, pH 8.0, 1% SDS, 1% sodium-deoxycholate, 20 mM EDTA, and 0.2 mM PMSF) per gram of tissue. Debris were pelleted by centrifugation at 1000*g* for 10 min.

Membrane Isolation by Differential Centrifugation

All extraction steps were done at 4°C. Different plant tissues were frozen in liquid nitrogen and ground in a mortar; 4 mL of extraction buffer (100 mM Tris-HCl, pH 7.5, 300 mM sucrose, 1 mM EDTA, and 0.1 mM PMSF) was added per gram of fresh tissue. The tissue was further ground, and debris was pelleted twice by centrifugations at 500*g* for 5 min. Organelles of high density were pelleted by centrifugation of the supernatant at 8000*g* for 15 min. The supernatant was further centrifuged at 100,000*g* for 2 hr to pellet microsomal membranes. The final supernatant corresponded to cytosol. The 100,000*g* pellets were resuspended in extraction buffer containing 0.4% SDS and 1% Triton X-100.

Protease Treatments

Seeds of transgenic Arabidopsis plants expressing phytohemagglutinin were germinated in liquid media (Bar-Peled et al., 1995). After 2 weeks of culture, 2 g of roots were ground in 5 mL of lysis buffer (50 mM Hepes-KOH, pH 6.8, 10 mM potassium acetate, 1 mM EDTA, 400 mM sucrose, 1 mM DTT, and 0.1 mM PMSF). Alternately, protoplasts were isolated from 50 mL of Arabidopsis cell suspensions, according to Bar-Peled and Raikhel (1997). Approximately 3 mL of packed protoplasts were resuspended in 6 mL of lysis buffer and lysed by more than eight passages through a 25⅝-gauge needle. After the root and cell suspension lysates were cleared at 1000*g*, they were spun at 8000*g*. The supernatant (S8) was saved. All proteases were from Sigma, and the stocks were prepared at 1 mg/mL in lysis buffer (pepsin, trypsin, and papain) or in protease K buffer (50 mM Tris-HCl, pH 7.5, and 1 mM $CaCl_2$). Aliquots of S8 (100 μL) were digested with 1 μg of protease in either the absence or presence of 1% Triton X-100 and were incubated on ice for various times from 0 to 30 min. At the end of each time point, 10 μg of leupeptin (Sigma) and 50 μL of boiling 3 × SDS sample buffer (188 mM Tris-HCl, pH 6.8, 30% glycerol, 3% SDS, and 3% β-mercaptoethanol) were added to each aliquot and boiled for an additional 5 min to destroy the protease. Equal volumes of each time point were separated by SDS-PAGE, blotted to nitrocellulose, and probed with antibodies.

Step Sucrose Gradients

The fractionation of cellular membranes was done at 4°C, based on the procedure of Gibeaut and Carpita (1994) and modified by M. Bar-Peled and N.V. Raikhel (unpublished data). Arabidopsis roots (1.5 g) were chopped with a razor blade and ground with glass beads in 6 mL of lysis buffer (50 mM Hepes-NaOH, pH 7.0, 13.5% [w/v] sucrose, and 10 mM NaCl) containing 0.5 mM PMSF, 1 mM DTT, and either 2 mM EDTA or 3 mM $MgCl_2$. The homogenate was passed four times through a 22.5-gauge needle, transferred to a centrifuge tube, and spun at 1000*g* for 10 min. The supernatant was collected and loaded on the top of a sucrose step gradient consisting of 2 mL of 56.0%, 5.84 mL of 40.0%, 4.66 mL of 33.5%, 4 mL of 26.5%, and 3.0 mL of 16% sucrose solution (w/v) in 10 mM Hepes-NaOH, pH 7.0, 10 mM potassium acetate, and either 2 mM EDTA or 5 mM $MgCl_2$. The gradient was centrifuged at 110,000*g* for 2 hr. Fractions of 1.4 mL were collected starting from the top of the gradient, trichloroacetic acid precipitated, and analyzed by SDS-PAGE.

Two-Phase Partitioning Method

The preparation of plasma membrane fractions from Arabidopsis cell suspension cultures was performed according to Daniels et al. (1994), whose protocol was based on Kjellbom and Larsson (1984). Repartitioning was done four times for both the plasma membrane and the intracellular membrane phase.

Protoplast Isolation and Vacuole Flotation

Protoplasts from Arabidopsis cell suspension cultures were prepared based on Axelos et al. (1992) with modifications. Arabidopsis cultures (100 mL) were filtered through a 1.5-mm mesh to remove large clumps. The cells were collected in a 50-μm nylon mesh and washed with 135 mL of B5 medium (3.2 g/L Gamborg's B5 medium and 2% sucrose [w/v], pH 5.7), followed by a 100-mL wash in protoplast medium (3.2 g/L Gamborg's B5 medium, 15.4% sucrose [w/v], pH 5.7). The cells were transferred into 15 mL of filtered (0.45 μm) enzyme solution (0.1 g of cytolase 345 L and 0.015 g of pectolyase Y23 in protoplast medium). After incubation for 3 hr under agitation (70 rpm) at room temperature in the dark, the protoplast solution was filtered through a 50-μm nylon sieve to remove undigested cells. Protoplasts were centrifuged in a Babcock bottle for 10 min (1200 rpm in a clinical centrifuge). The floating protoplasts were collected, diluted in wash buffer (0.4 M betaine, 10 mM $CaCl_2 \cdot 2H_2O$, and 3 mM Mes, pH 5.7), and centrifuged for 10 min (80*g*). The protoplast pellet was resuspended in prewarmed (37°C) lysis buffer (0.2 M mannitol, 20 mM EDTA, 2 mM DTT, 5 mM Hepes, and 10% Ficoll type 400 [Sigma], pH 8.0). Vacuoles were isolated according to Höfte et al. (1991).

Electron Microscopy

For electron microscopy, the procedure described by Tokuyasu (1980) was used. Frozen thin sections were prepared from Arabidopsis root tips. Seeds were soaked in distilled water for 1 hr at room temperature and germinated on humidified filter papers on Petri dishes. The plates were kept at a vertical position and incubated at 20°C in the presence of light. After 2 to 3 days, the root tips were immersed in freshly prepared 2% (v/v) formaldehyde and 0.1 M sodium phosphate buffer, pH 7.4, for 6 hr at room temperature. The specimens were rinsed in 0.1 M phosphate buffer and cryoprotected by infiltration in a 0.1 M phosphate buffer solution containing 2.3 M sucrose and 10% (w/v) polyvinylpyrrolidone K 15 (Fluka, Buchs, Switzerland) for 2 hr at room temperature (Tokuyasu, 1989). Root tips were mounted individually onto an aluminium specimen holder and directly frozen in liquid nitrogen. Ultrathin cryosections (60 to 80 nm) were cut with a diamond knife (Diatome, Biel, Switzerland) at −120°C on a microtome (model UCT; Leica, Heerbrugg, Switzerland) equipped with an FCS cryo-attachment housing an antistatic device (Diatome). Cryosections were transferred on formvar/carbon–coated nickel grids that were ionized before use. The grids were floated on solutions. After blocking in 5% (v/v) newborn calf serum in PBS, sections were incubated with primary antibodies raised against AtPEP12p (1:20 for 4 hr at 20°C), γ-TIP antibodies (Marty-Mazars et al., 1995; 1:500 for 1 hr at 20°C), or corresponding preimmune sera. Excess primary antibodies were removed by multiple washes in 1% (v/v) newborn calf serum in PBS. AtPEP12p antibodies and the related control with preimmune serum were detected by first labeling with biotinylated goat anti–rabbit IgG (Amersham) for 1 hr at 20°C, followed by incubation with streptavidin conjugated to 10-nm colloidal gold particles (30 min at 4°C in the dark). Detection of γ-TIP and the related control with preimmune serum were done using a secondary antibody linked directly to 5-nm colloidal gold particles (Amersham). The grids were washed in double distilled water and stained according to Griffiths et al. (1983). The sections were observed with an electron microscope (model H600; Hitachi, Tokyo, Japan) operating at 75 kV.

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REFERENCES

Aalto, M.K., Ronne, H., and Keränen, S. (1993). Yeast syntaxins Sso1p and Sso2p belong to a family of related membrane proteins that function in vesicular transport. EMBO J. **12,** 4095–4104.

Acharya, U., Jacobs, R., Peters, J.-M., Watson, N., Farquhar, M., and Malhotra, V. (1995). The formation of Golgi stacks from vesiculated Golgi membranes requires two distinct fusion events. Cell **82,** 895–904.

Axelos, M., Curie, C., Mazzolini, L., Bardet, C., and Lescure, B. (1992). A protocol for transient gene expression in *Arabidopsis thaliana* protoplasts isolated from cell suspension cultures. Plant Physiol. Biochem. **30,** 123–128.

Bar-Peled, M., and Raikhel, N.V. (1996). A method for isolation and purification of specific antibodies to a protein fused to the GST. Anal. Biochem. **241,** 140–142.

Bar-Peled, M., and Raikhel, N.V. (1997). Characterization of AtSEC12 and AtSAR1, proteins likely involved in ER and Golgi transport. Plant Physiol. **114,** in press.

Bar-Peled, M., da Silva Conceição, A., Frigerio, L., and Raikhel, N.V. (1995). Expression and regulation of *aERD2*, a gene encoding the KDEL receptor homolog in plants, and other genes encoding proteins involved in ER–Golgi vesicular trafficking. Plant Cell **7,** 667–676.

Bassham, D.C., Gal, S., da Silva Conceição, A., and Raikhel, N.V. (1995). An *Arabidopsis* syntaxin homologue isolated by functional complementation of a yeast *pep12* mutant. Proc. Natl. Acad. Sci. USA **92,** 7262–7266.

Becherer, K.A., Rieder, S.E., Emr, S.D., and Jones, E.W. (1996). A novel syntaxin homologue, Pep12p, required for the sorting of lumenal hydrolases to the lysosome-like vacuole in yeast. Mol. Cell. Biol. **7,** 579–594.

Bennett, M.K. (1995). SNAREs and the specificity of transport vesicle targeting. Curr. Opin. Cell Biol. **7,** 581–586.

Bennett, M.K., Calakos, N., and Scheller, R.H. (1992a). Syntaxin: A synaptic protein implicated in the docking of synaptic vesicles at presynaptic active zones. Science **257,** 255–259.

Bennett, M.K., Calakos, N., Kreiner, T., and Scheller, R.H. (1992b). Synaptic vesicle membrane proteins interact to form a multimeric complex. J. Cell Biol. **116,** 761–775.

Bennett, M.K., Garcia-Arrarás, J.E., Elferink, L.A., Peterson, K., Fleming, A.M., Hazuka, C.D., and Scheller, R.H. (1993). The syntaxin family of vesicular transport receptors. Cell **74,** 863–873.

Bent, A.F., Kunkel, B.N., Dahlbeck, D., Brown, K.L., Schmidt, R., Giraudat, J., Leung, J., and Staskawicz, B.J. (1994). *RPS2* of *Arabidopsis thaliana*: A leucine-rich repeat class of plant disease resistance genes. Science **265,** 1856–1860.

Burd, C.G., Mustol, P.A., Schu, P.V., and Emr, S.D. (1996). A yeast protein related to a mammalian Ras-binding protein, Vps9p, is required for localization of vacuolar proteins. Mol. Cell. Biol. **16,** 2369–2377.

Calakos, N., Bennett, M.K., Peterson, K.E., and Scheller, R.H. (1994). Protein–protein interactions contributing to the specificity of intracellular vesicular trafficking. Science **263,** 1146–1149.

Chrispeels, M.J., and Raikhel, N.V. (1992). Short peptide domains target proteins to the plant vacuoles. Cell **68,** 613–616.

Cowles, C.R., Emr, S.D., and Horazdovsky, B.F. (1994). Mutations in the *VPS45* gene, a *SEC1* homologue, result in vacuolar protein sorting defects and accumulation of membrane vesicles. J. Cell Sci. **107,** 3449–3459.

Daniels, M.J., Mirkov, T.E., and Chrispeels, M.J. (1994). The plasma membrane of *Arabidopsis thaliana* contains a mercury-insensitive aquaporin that is a homolog of the tonoplast water channel protein TIP. Plant Physiol. **106,** 1325–1333.

Gal, S., and Raikhel, N.V. (1993). Protein sorting in the endomembrane system of plant cells. Curr. Opin. Cell Biol. **5,** 636–640.

Gibeaut, D.M., and Carpita, N.C. (1994). Improved recovery of (1→3),(1→4)-β-D-glucan synthase activity from Golgi apparatus of *Zea mays* (L.) using differential flotation centrifugation. Protoplasma **180,** 92–97.

Graham, T.R., and Emr, S.D. (1991). Compartmental organization of Golgi-specific protein modification and vacuolar protein sorting events defined in a yeast *sec18* (NSF) mutant. J. Cell Biol. **114,** 207–218.

Griffiths, G., Simons, K., Warren, G., and Tokuyasu, K.T. (1983). Immunoelectron microscopy using thin, frozen sections: Application to studies of the intracellular transport of Semliki forest virus spike glycoproteins. Methods Enzymol. **96,** 466–485.

Haas, A., and Wickner, W. (1996). Homotypic vacuole fusion requires Sec17p (yeast α-SNAP) and Sec18p (yeast NSF). EMBO J. **15,** 3296–3305.

Herman, E.M., Li, X., Su, R.T., Larsen, P., Hsu, H.-t., and Sze, H. (1994). Vacuolar-type H^+-ATPases are associated with the endoplasmic reticulum and provacuoles of root tip cells. Plant Physiol. **106,** 1313–1324.

Herman, P.K., Stack, J.H., De Modena, J.A., and Emr, S.D. (1991). A novel protein kinase homolog essential for protein sorting to the yeast lysosome-like vacuole. Cell **64,** 425–437.

Höfte, H., Faye, L., Dickinson, C., Herman, E.M., and Chrispeels, M.J. (1991). The protein body proteins phytohemagglutinin and tonoplast intrinsic protein are targeted to vacuoles in leaves of transgenic tobacco. Planta **184,** 431–437.

Hoh, B., Hinz, G., Jeong, B.-K., and Robinson, D.G. (1995). Protein storage vacuoles form de novo during pea cotyledon development. J. Cell Sci. **108,** 299–310.

Hohl, I., Robinson, D.G., Chrispeels, M.J., and Hinz, G. (1996). Transport of storage proteins to the vacuole is mediated by vesicles without a clathrin coat. J. Cell Sci. **109,** 2539–2550.

Horazdovsky, B.F., Busch, G.R., and Emr, S.D. (1994). *VPS21* encodes a Rab5-like GTP binding protein that is required for the sorting of yeast vacuolar proteins. EMBO J. **13,** 1297–1309.

Ibaraki, K., Horikawa, H.P.M., Morita, T., Mori, H., Sakimura, K., Mishina, M., Saisu, H., and Abe, T. (1995). Identification of four different forms of syntaxin 3. Biochem. Biophys. Res. Commun. **211,** 997–1005.

Ikonen, E., Tagaya, M., Ullrich, O., Montecucco, C., and Simons, K. (1995). Different requirements for NSF, SNAP, and Rab proteins in apical and basolateral transport in MDCK cells. Cell **81,** 571–580.

Jones, E.W. (1976). Proteinase mutants of *Saccharomyces cerevisiae*. Genetics **85,** 23–33.

Kjellbom, P., and Larsson, C. (1984). Preparation and polypeptide composition of chlorophyll-free plasma membranes from leaves of light-grown spinach and barley. Physiol. Plant. **62,** 501–509.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227,** 680–685.

Latterich, M., Fröhlich, K.-U., and Schekman, R. (1995). Membrane fusion and the cell cycle: Cdc48p participates in the fusion of ER membranes. Cell **82,** 885–893.

Lord, J.M. (1987). Isolation of endoplasmic reticulum: General principles, enzymatic markers, and endoplasmic reticulum–bound polysomes. Methods Enzymol. **148,** 576–584.

Lukowitz, W., Mayer, U., and Jürgens, G. (1996). Cytokinesis in the Arabidopsis embryo involves the syntaxin-related *KNOLLE* gene product. Cell **84,** 61–71.

Marty-Mazars, D., Clémencet, M.-C., Cozolme, P., and Marty, F. (1995). Antibodies to the tonoplast from the storage parenchyma cells of beetroot recognize a major intrinsic protein related to TIPs. Eur. J. Cell Biol. **66,** 106–118.

Matsuoka, K., Bassham, D.C., Raikhel, N.V., and Nakamura, K. (1995). Different sensitivity to wortmannin of two vacuolar sorting signals indicates the presence of distinct sorting machineries in tobacco cells. J. Cell Biol. **130,** 1307–1318.

Mayer, A., Wickner, W., and Haas, A. (1996). Sec18p (NSF)-driven release of Sec17p (a-SNAP) can precede docking and fusion of yeast vacuoles. Cell **85,** 83–94.

Mellman, I. (1995). Enigma variations: Protein mediators of membrane fusion. Cell **82,** 869–872.

Melroy, D.L., and Herman, E.M. (1991). TIP, an integral membrane protein of the protein-storage vacuoles of the soybean cotyledon, undergoes developmentally regulated membrane accumulation and removal. Planta **184,** 113–122.

Nakamura, K., and Matsuoka, K. (1993). Protein targeting to the vacuole in plant cells. Plant Physiol. **101,** 1–5.

Paris, N., Stanley, C.M., Jones, R.L., and Rogers, J.C. (1996). Plant cells contain two functionally distinct vacuolar compartments. Cell **85,** 563–572.

Pedrazzini, E., and Vitale, A. (1995). The binding protein, BiP, and the synthesis of secretory proteins. Plant Physiol. Biochem. **34,** 207–216.

Piper, R.C., Whitters, E.A., and Stevens, T.H. (1994). Yeast Vps45p is a Sec1p-like protein required for the consumption of vacuole-targeted, post–Golgi transport vesicles. Eur. J. Cell Biol. **65,** 305–318.

Rabouille, C., Levine, T.P., Peters, J.-M., and Warren, G. (1995). An NSF-like ATPase, p97, and NSF mediate cisternal regrowth from mitotic Golgi fragments. Cell **82,** 905–914.

Raymond, C.K., O'Hara, P.J., Eichinger, G., Rothman, J.H., and Stevens, T.H. (1990). Molecular analysis of the yeast VPS3 gene and the role of its product in vacuolar protein sorting and vacuolar segregation during the cell cycle. J. Cell Biol. **111,** 877–892.

Rothman, J.E., and Wieland, F.T. (1996). Protein sorting by transport vesicles. Science **272,** 227–234.

Schu, P.V., Takegawa, K., Fry, M.J., Stack, J.H., Waterfield, M.D., and Emr, S.D. (1993). Phosphatidylinositol 3-kinase encoded by yeast *VPS34* gene essential for protein sorting. Science **260,** 88–91.

Söllner, T., Bennett, M.K., Whiteheart, S.W., Scheller, R.H., and Rothman, J.E. (1993a). A protein assembly–disassembly pathway *in vitro* that may correspond to sequential steps of synaptic vesicle docking, activation and fusion. Cell **75,** 409–418.

Söllner, T., Whiteheart, S.W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P., and Rothman, J.E. (1993b). SNAP receptors implicated in vesicle targeting and fusion. Nature **362,** 318–324.

Tokuyasu, K.T. (1980). Immunochemistry on ultrathin frozen sections. Histochem. J. **12,** 381–403.

Tokuyasu, K.T. (1989). Use of poly(vinylpyrrolidone) and poly(vinyl alcohol) for cryoultramicrotomy. Histochem. J. **21,** 163–171.

Ueda, T., Anai, T., Tsukaya, H., Hirata, A., and Uchimiya, H. (1996). Characterization and subcellular localization of a small GTP-binding protein (*Ara-4*) from *Arabidopsis*: Conditional expression under control of the promoter of the gene for heat-shock protein HSP81-1. Mol. Gen. Genet. **250,** 533–539.

von Schaewen, A., Sturm, A., O'Neill, J., and Chrispeels, M.J. (1993). Isolation of a mutant *Arabidopsis* plant that lacks N-acetylglucosaminyl transferase I and is unable to synthesize Golgi-modified complex N-linked glycans. Plant Physiol. **102,** 1109–1118.

Weisman, L.S., and Wickner, W. (1992). Molecular characterization of *VAC1*, a gene required for vacuole inheritance and vacuole protein sorting. J. Biol. Chem. **267,** 618–623.