The Plant Vacuolar Sorting Receptor AtELP Is Involved in Transport of NH_2-terminal Propeptide-containing Vacuolar Proteins in *Arabidopsis thaliana*

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Abstract. Many soluble plant vacuolar proteins are sorted away from secreted proteins into small vesicles at the trans-Golgi network by transmembrane cargo receptors. Cleavable vacuolar sorting signals include the NH_2-terminal propeptide (NTPP) present in sweet potato sporamin (Spo) and the COOH-terminal propeptide (CTPP) present in barley lectin (BL). These two proteins have been found to be transported by different mechanisms to the vacuole. We examined the ability of the vacuolar cargo receptor AtELP to interact with the sorting signals of heterologous and endogenous plant vacuolar proteins in mediating vacuolar transport in *Arabidopsis thaliana*. AtELP extracted from microsomes was found to interact with the NTPPs of barley aleurain and Spo, but not with the CTPPs of BL or tobacco chitinase, in a pH-dependent and sequence-specific manner. In addition, EM studies revealed the colocalization of AtELP with NTPP-Spo at the Golgi apparatus, but not with BL-CTPP in roots of transgenic *Arabidopsis* plants. Further, we found that AtELP interacts in a similar manner with the NTPP of the endogenous vacuolar protein AtALEU (*Arabidopsis thaliana* Aleu), a protein highly homologous to barley aleurain. We hypothesize that AtELP functions as a vacuolar sorting receptor involved in the targeting of NTPP-, but not CTPP-containing proteins in *Arabidopsis*.

Key words: protein traffic • Golgi apparatus • COOH-terminal propeptide • plant vacuole barley aleurain

Introduction

In mature plant cells, the vacuole is the largest of the membrane-bound organelles of the endomembrane system. Plant vacuoles perform a diverse set of functions that are essential for the regulation and maintenance of plant growth and development (reviewed in Marty, 1999). Unlike yeast vacuoles or mammalian lysosomes, the plant vacuole often serves as both a lytic compartment for the degradation of materials and as a storage area for proteins. The stored proteins must be kept separate from degrading proteases until conditions in the plant require their mobilization. Recent evidence suggests that cells of some plant tissues have multiple vacuoles that differ in size, shape, content, and function (reviewed in Vitale and Raikhel, 1999), perhaps making possible physical separation of proteins and proteases. The proper identity and function of these different vacuoles is maintained by the transport of appropriate membrane and soluble proteins, which serve as markers for each type of vacuole.

Many soluble plant vacuolar proteins are sorted away from proteins destined for secretion at the trans-Golgi network (TGN)[1], a process that requires the presence of positive sorting signals on the vacuolar proteins. Three types

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[1]*Abbreviations used in this paper:* AtALEU, *Arabidopsis thaliana* Aleu; barley Aleu, barley aleurain; BL, barley lectin; CCVs, clathrin-coated vesicles; CTPP, COOH-terminal propeptide; LV, lytic vacuole; Mt, mutant; NTPP, NH_2-terminal propeptide; ORF, open reading frame; PI, phosphatidylinositol; PVC, prevacuolar compartment; Spo, sporamin; TGN, trans-Golgi network; TobChit, tobacco chitinase; Wt, wild-type.

of sorting signals have been described for soluble vacuolar proteins in plants (reviewed in Matsuoka and Neuhaus, 1999; Vitale and Raikhel, 1999). Some proteins, such as barley aleurain (barley Aleu) and sweet potato sporamin (Spo), contain a cleavable NH_2-terminal propeptide (NTPP) that functions as a sorting signal; others (e.g., barley lectin [BL] and tobacco chitinase A [TobChit]) contain a cleavable COOH-terminal propeptide (CTPP). Finally, some mature proteins, such as phytohemagglutinin and legumin, contain an internal targeting determinant. These targeting signals appear to be specific to plants because plant vacuolar proteins expressed in yeast are targeted to the yeast vacuole by a mechanism that is independent of the plant sorting signals (Matsuoka and Nakamura, 1992; Chao and Etzler, 1994; Gal and Raikhel, 1994). Targeting of mammalian lysosomal proteins also differs: sorting signals are not encoded by the amino acid sequence, but rather involve posttranslationally added sugar modifications, such as phosphomannosyl residues (reviewed in Kornfeld, 1992).

NTPP signals contain an NPIR consensus amino acid motif that is necessary for targeting Spo to the vacuole (Nakamura and Matsuoka, 1993; Matsuoka et al., 1995). In contrast to the NTPP signals, no consensus sequence has yet been identified for the CTPP targeting domains. These domains are often enriched in hydrophobic amino acids and it is hypothesized that rather than sequence specificity, a common structural feature may serve as the sorting signal in the CTPPs (reviewed in Matsuoka and Neuhaus, 1999). Recent results indicate that the CTPP- and NTPP-dependent pathways are biochemically distinct (Matsuoka et al., 1995; Frigerio et al., 1998). The transport of BL by the CTPP-mediated pathway was found to be sensitive to wortmannin, an inhibitor of phosphatidylinositol (PI) 3-kinases and phospholipid synthesis in plants (Matsuoka et al., 1995). However, the transport of Spo by the NTPP-mediated pathway is not affected by wortmannin. NTPP-containing proteins are thought to be transported from the Golgi apparatus to the lytic vacuole (LV) in clathrin-coated vesicles (CCVs) via the prevacuolar compartment (PVC), and CTPP-containing proteins are transported to a vacuole, distinct from the LV (reviewed in Paris et al., 1996; Vitale and Raikhel, 1999). Similar to yeast and mammalian cells, the transport of proteins to the plant vacuole is saturable, indicating the involvement of sorting receptors that might interact with these signals at the TGN (reviewed by Vitale and Raikhel, 1999).

We have isolated and characterized AtELP, a potential vacuolar sorting receptor that shares many features common to several eukaryotic sorting receptors (Ahmed et al., 1997). AtELP is not homologous to any mammalian or yeast proteins found in the database (Ahmed et al., 1997; Paris et al., 1997). It is enriched in plant CCVs, and probably recycles between the TGN and the PVC (Ahmed et al., 1997; Sanderfoot et al., 1998). Further, the AtELP cytoplasmic tail preferentially interacts with the mammalian TGN-specific, clathrin-associated AP-1 adaptor complex of proteins in vitro (Sanderfoot et al., 1998). Its biochemical properties and subcellular distribution thus suggest that AtELP may play a role in targeting proteins to the plant vacuole. Although AtELP-related proteins have been identified from several plant species (Paris et al., 1997; Shimada et al., 1997; Miller et al., 1999), their interactions with the various types of vacuolar sorting signals vary, depending on the tissue source. For example, BP-80 from pea interacts with both the barley Aleu- and Spo-NTPPs (Kirsch et al., 1994) and the COOH-terminal sorting signal of Brazil nut 2S albumin (Kirsch et al., 1996); its homologue in tobacco cells interacts with the Na-PI precursor protein containing a COOH-terminal vacuolar sorting signal (Miller et al., 1999). However, no direct evidence indicates that the interaction of the tobacco homologue of BP-80 with Na-PI is specifically mediated by the COOH-terminal sorting signal of the protein. Moreover, there is no evidence that Na-PI in tobacco cells reaches the vacuole using the wortmannin-sensitive CTPP pathway.

Here, we have investigated further the role of AtELP in vacuolar targeting using a biochemical assay and have found that it interacts with the NTPP-, but not CTPP-based vacuolar sorting signals, in a sequence-specific and pH-dependent manner. Moreover, using immunoelectron microscopy, we have found that AtELP colocalizes with a vacuolar cargo containing NTPP, but not with that containing CTPP. These results strongly support the proposed function of AtELP as a vacuolar sorting receptor that would select NTPP-containing proteins at the TGN for sorting into CCVs for eventual delivery to the vacuole in *Arabidopsis thaliana*.

Materials and Methods

Cloning of Arabidopsis thaliana Aleu (AtALEU)

A search of the *Arabidopsis* expressed sequence tag (EST) database using the barley Aleu amino acid sequence (Rogers et al., 1985) and the Blast program (Altschul et al., 1990) identified an EST whose predicted amino acid sequence encoded an open reading frame (ORF) of 358 residues that is 70% identical to barley Aleu (data not shown). This clone (GenBank/EMBL/DDBJ accession number AF233883) containing the ORF of 358 amino acids was termed *AtALEU*.

Antibody Production

A polyclonal antibody (α-AtALEU) against AtALEU was produced by immunizing rabbits with a fusion protein encoding six histidines, followed by amino acids 228–358 of the predicted AtALEU ORF. The fusion protein was constructed in pET28a (Novagen), induced in *Escherichia coli* with isopropyl-1-thio-β-D-galactopyranoside, and purified by Ni^{2+}-Sepharose affinity chromatography. Affinity-purified AtALEU antiserum was prepared according to previously described procedures (Bassham and Raikhel, 1998) and used in both immunoblotting and EM. AtELP (Ahmed et al., 1997), Spo (Matsuoka et al., 1995), BL (Dombrowski et al., 1993), and AtSEC12 (Bar-Peled and Raikhel, 1997) rabbit antisera and preimmune sera have been previously described.

Plant Material

The full-length BL-CTPP (Wilkins et al., 1990) and NTPP-Spo (Matsuoka and Nakamura, 1991) cDNA clones were transformed into *Arabidopsis thaliana* ecotypes RLD and Columbia plants, respectively, in the pGA643 binary vector under the transcriptional control of the CaMV 35S promoter. The transformation was carried out with *Agrobacterium tumefaciens* strain GV3101, PMP90 using vacuum infiltration as described by Bent et al. (1994). Transformants were selected on kanamycin and the presence of Spo and BL was detected in several independent lines by protein gel blot analysis using α-Spo or α-BL antiserum (Dombrowski et al., 1993; Matsuoka et al., 1995). *Arabidopsis* ecotype Columbia cell suspension cultures were maintained as previously described (Ahmed et al., 1997).

Affinity Column Chromatography

The affinity column chromatography procedures used were adapted from previously described protocols (Kirsch et al., 1994, 1996). To prepare affinity columns, peptides were commercially synthesized at Research Genetics Inc. to >85% purity. For the NTPP peptides, a cysteine residue was added at the COOH-terminal end of each peptide for subsequent chemical coupling to Sulfolink agarose beads (Pierce Chemical Co.) according to the manufacturer's protocols. The BL-CTPP peptide was coupled to Affigel-15 beads (BioRad) according to the manufacturer's protocols. For the putative NTPP signal of AtALEU, sequences for the peptides used were designed based on the exact number of residues both upstream and downstream of the NPIR motif (amino acids 22–42), consistent with the barley probarley Aleu sorting signal.

Vacuole Purification

Vacuoles were purified from *Arabidopsis* cell suspension culture according to Gomez and Chrispeels (1993), with modifications. For biochemical analyses, both protoplasts and purified vacuoles were first briefly centrifuged in a microfuge. The resulting supernatant was discarded and the pelleted protoplasts and vacuoles were lysed with protein extraction buffer: 50 mM $NaPO_4$, pH 7.0, 10 mM EDTA, 1% Triton X-100, 1% Sarkosyl, 1 mM PMSF. The solubilized materials were separated by centrifugation at 13,000 *g* for 10 min at 4°C. The resulting supernatant containing total protein from protoplasts and vacuoles was analyzed by either immunoblotting, using antisera specific to markers for different subcellular organelles, or for the presence of vacuolar-specific enzyme activities of α-mannosidase and acid phosphatase as below.

Vacuolar Enzyme Activity Assays

α-Mannosidase and acid phosphatase activities were measured using 4-methylumbelliferyl–linked substrates with modifications of previously described procedures (Reilly et al., 1996; Vazquez-Reyna et al., 1999). Reactions were carried out at 37°C for 1 h and quenched with 1.5 ml of 0.25 M Na_2CO_3. Fluorescence was measured on a Hitachi F-2000 Fluorescence Spectrophotometer using an excitation wavelength of 365 nm, detecting the emission at 455 nm. The activities were calculated in mol/liter of methylumbelliferone released per hour per microgram of protein. The ratios of the activity for each enzyme in vacuoles with respect to protoplasts were compared (with protoplasts = 1).

Electron Microscopy

The procedures used for immunogold EM of ultrathin plastic sections were as previously described (Zheng et al., 1999), with some minor modifications. In the quantitative analysis, all membrane structures that were found within 50–100 nm of the Golgi apparatus or the vacuole were considered. In total, ~180–220 gold particles were counted for each of the labeled antibodies over 25–30 independent Golgi apparatuses that were analyzed in three independent experiments. Finally, the percentage of total gold particles that were found over a specific compartment was calculated, together with the percentage of colocalization of AtELP with either Spo or BL at the Golgi apparatus or other structures near the vacuole (Tables I, II, and III). Ultrathin cryosections of *Arabidopsis* roots were prepared and immunogold-labeling experiments were carried out using previously described procedures (Sanderfoot et al., 1998).

Online Supplemental Material

Detailed methods for preparation of microsomes, affinity chromatography, vacuole preparation, vacuolar enzyme assays, and EM. Online supplemental materials are available at http://www.jcb.org/cgi/content/full/149/7/1335/DC1.

Supplemental Figure S1. Immunogold-labeling of transgenic *Arabidopsis* root tissue using preimmune serum for BL and Spo.

Supplemental Figure S2. Immunogold-labeling of AtALEU and Spo in *Arabidopsis* root tissues.

Results

AtELP Interacts with the Vacuolar Targeting Signals of Barley Aleu and Spo in a pH-dependent Manner

We have reported the biochemical characterization of a sorting receptor-like protein from *Arabidopsis*, AtELP, that is associated with CCVs and resides on the TGN and on a PVC (Ahmed et al., 1997; Sanderfoot et al., 1998). We proposed a potential role for AtELP in protein transport along the vacuolar pathway in *Arabidopsis*. Here, we investigated the ability of AtELP to interact with peptides representing the targeting determinants of several plant vacuolar proteins (Table I), in an in vitro binding assay. AtELP, present in a detergent extract prepared from microsomes of *Arabidopsis* cells, was retained on the peptide affinity columns containing the wild-type (Wt)-NTPP-barley Aleu and Wt-NTPP-Spo vacuolar sorting signals at neutral pH and were subsequently eluted with an acidic buffer (Fig. 1 A). These peptides contain a consensus NPIR motif that is an important component of the NTPP sorting signals of barley Aleu and Spo. In vivo, either the deletion of the NPIR sequence or the substitution of Gly for Ile in the proSpo vacuolar sorting signal results in 90% secretion of Spo to the culture media (Nakamura and Matsuoka, 1993). In probarley Aleu, sequences in addition to

Table I. NTPP- and CTPP-containing Plant Vacuolar Sorting Signals

Propeptide	Sequence	Sufficient*	Reference
Barley Aleu			
Wt-NTPP-barley Aleu	SSSFADSNPIRPVTDRAASTYC‡	Yes	Holwerda et al., 1992
Mt-NTPP-barley Aleu	SSSFADSNPGRPVTDRAASTYC‡	N/T§	
Spo			
Wt-NTPP-Spo	SRFNPIRLPTC‡	Yes	Matsuoka et al., 1995
Mt-NTPP-Spo	SRFNPGRLPTC‡	No	Matsuoka et al., 1995
BL			
Wt-BL-CTPP	VFAEAIAANSTLVAE	Yes	Vitale and Raikhel, 1999
TobChit			
Wt-TobChit-CTPP	GLLVDTM	Yes	Neuhaus and Rogers, 1998

Wt and Mt peptide sequences used in this study. These sequences correspond to either the NTPP or CTPP vacuolar targeting propeptides from each of the indicated vacuolar proteins.
*Indicates whether the propeptide can redirect a reporter or secreted protein to the vacuole.
‡A cysteine residue was added to each of the indicated peptides at the COOH-terminal end for coupling to the affinity matrix.
§The effect of the Gly to Ile substitution has not been tested for the barley Aleu peptide.

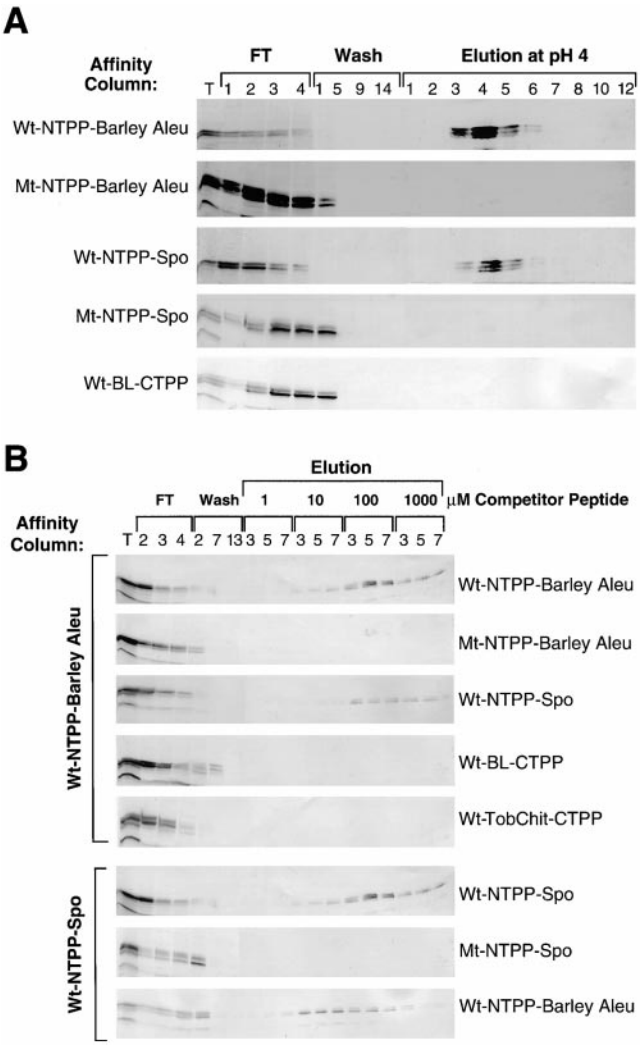

Figure 1. Binding of AtELP to vacuolar targeting signals is both pH-dependent and sequence-specific. A, SDS-PAGE and Western analysis of AtELP binding to the Wt-NTPP–targeting propeptides of barley Aleu and Spo. Binding assay with peptide affinity columns was carried out as described in Materials and Methods. The bound proteins were eluted under acidic conditions (pH 4). Fractions were collected for: T, total extract loaded onto each affinity column; FT, flow through; Wash, washes with binding buffer; Elution, protein eluted under low pH (4.0). B, Binding of AtELP to the NTTPs is sequence-specific. The specificity of AtELP binding to the Wt-NTPP-barley Aleu and Wt-NTPP-Spo propeptides was determined in a competition assay. The extract in A was bound to either the Wt-NTPP-barley Aleu or Wt-NTPP-Spo affinity column as described in A. The bound proteins were eluted using increasing concentrations (1–1,000 μM) of the indicated peptides as competitors in the same binding buffer. For each of the flow through (FT), wash, and elution steps, fractions were collected. The indicated fraction numbers for both A and B were analyzed by immunoblotting with α-AtELP antiserum.

the NPIR sequence contribute to the maximum level of vacuolar transport of barley Aleu in vivo (Holwerda et al., 1992). Accordingly, AtELP was not retained by the mutant (Mt)-NTPP-barley Aleu or Mt-NTPP-Spo affinity column, where the Ile in the NPIR motif present in the barley Aleu- and Spo-targeting sequences was changed to a Gly (Fig. 1 A). An affinity column representing the BL-CTPP retained no detectable amounts of AtELP from the detergent extract, suggesting that AtELP does not interact with the CTPP-sorting signal of BL. In immunoblot analysis of our binding experiments, using AtELP antibodies, we consistently detected three closely migrating polypeptides that bound to the Wt-NTPP-barley Aleu and Wt-NTPP-Spo affinity columns. These three polypeptides demonstrated equal affinity for the two different NTPPs in the competition assays described below. They could represent different posttranslational modifications of AtELP, or different isoforms of AtELP that are immunologically related proteins having biochemical properties similar to AtELP. Moreover, Ahmed et al. (1997) showed that these three polypeptides have identical tissue and subcellular distribution.

Sequence Specificity of AtELP Binding

To further investigate the specificity of AtELP's interaction with barley Aleu and Spo NTPPs, we carried out competition studies using six different peptides (Fig. 1 B). In these experiments, the Wt-NTPP-barley Aleu and Wt-NTPP-Spo peptides successfully competed for the binding of AtELP to the corresponding Wt-NTPP-barley Aleu or Wt-NTPP-Spo peptide columns at ~100 μM concentration. In addition, the Wt-NTPP-barley Aleu peptide competed for binding to the Wt-NTPP-Spo peptide. However, AtELP appeared to have an approximate tenfold higher affinity for the Wt-NTPP-barley Aleu peptide than for the Wt-NTPP-Spo peptide. In the presence of a 10-μM concentration of the Wt-NTPP-barley Aleu peptide, the majority of AtELP retained on the Wt-NTPP-Spo affinity column was eluted from the column. In contrast, a tenfold higher concentration (100 μM) of the Wt-NTPP-Spo peptide was required to elute a similar amount of AtELP retained on the Wt-NTPP-barley Aleu affinity column. This difference in the affinity of AtELP for the Wt-NTPP-barley Aleu may reflect the involvement of additional sorting determinants in the NTPP (see Discussion). The Mt-NTPP-barley Aleu or the Mt-NTPP-Spo peptides did not compete for binding. As opposed to the Spo and barley Aleu NTPPs, peptides corresponding to the vacuolar targeting sequences of the BL-CTPP or TobChit-CTPP did not compete for binding, at concentrations up to 1,000 μM (Fig. 1 B). Our results indicate that AtELP interacts in vitro, with the two NTPP-, but not with the CTPP-containing sorting signals in a pH-dependent manner. In addition, its interactions with these signals are dependent on the NPIR motif present in the peptides, which is necessary in vivo for their proper targeting to the plant vacuole.

AtELP Colocalizes with NTPP-Spo, but Not BL-CTPP, Vacuolar Cargo Protein in Transgenic Arabidopsis Roots

We have demonstrated that the vacuolar reporter proteins Spo and BL are transported to the vacuole by distinct pathways based on sensitivity to wortmannin (Matsuoka et al., 1995). In transgenic tobacco cells, vacuolar transport of BL-CTPP is inhibited by wortmannin, but the transport of NTPP-Spo is not. In this study, we have therefore used Spo and BL as reporters for the NTPP- and CTPP-mediated vacuolar sorting pathways in *Arabidopsis*, respectively.

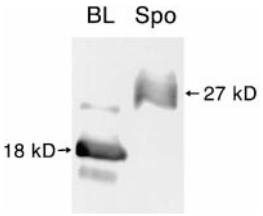

Figure 2. Western analysis of BL and Spo in transgenic *Arabidopsis* root tissue. Total proteins from roots of 2-wk-old seedlings were isolated and analyzed by SDS-PAGE, followed by immunoblotting using the α-BL or α-Spo antiserum. Polypeptides of 18 kD and 27 kD corresponding to the BL and Spo proteins, respectively, were recognized by the corresponding antiserum.

To use NTPP-Spo and BL-CTPP as vacuolar reporters in *Arabidopsis*, we obtained separate lines of transgenic plants expressing either Spo or BL as described in Materials and Methods. Expression of the reporter proteins was first analyzed by Western blot using α-Spo or α-BL antisera (Fig. 2). Both antisera detected polypeptides with an apparent molecular mass of 18 and 27 kD, corresponding to mature BL and Spo, respectively, as previously reported in tobacco (Schroeder et al., 1993). To determine whether both Spo and BL are transported to the vacuole, we examined ultrathin sections of roots by EM and immunocytochemical analysis. Electron-dense protein aggregates stained with either α-BL (Fig. 3 A) or α-Spo (Fig. 3 B) antiserum were seen predominantly in the vacuole. Both antisera showed some staining in the Golgi apparatus and structures near the vacuole, consistent with the pathways followed by the two reporters en route to the vacuole. Weak and nonspecific background labeling was detected in parallel experiments using preimmune serum for either antibody (Supplemental Figure S1, A and B). These results indicate that both Spo and BL are correctly transported to the vacuole in transgenic *Arabidopsis* plants.

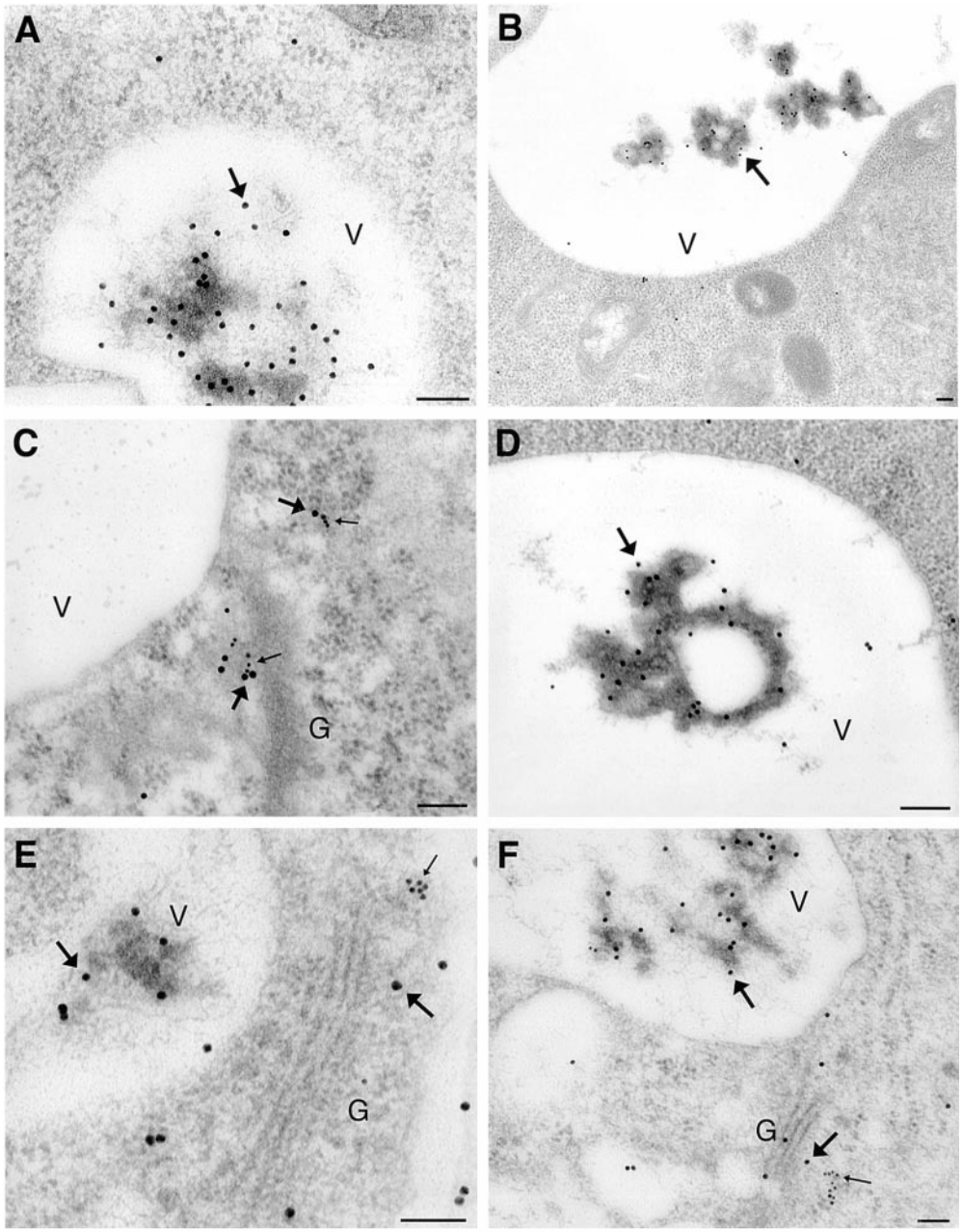

Figure 3. AtELP colocalizes with NTPP-Spo, but not with BL-CTPP in transgenic *Arabidopsis* roots. Immunogold labeling of BL (A) and Spo (B). Ultrathin sections of transgenic *Arabidopsis* roots expressing BL or Spo were treated with α-BL (A) or α-Spo (B) antiserum and the bound antibodies were visualized with protein A coupled to gold particles. Arrows represent 15-nm BL- or 10-nm Spo-associated gold particles. C–F, In double immunogold labeling experiments of AtELP and Spo or BL, thin sections of transgenic *Arabidopsis* roots expressing Spo (C and D) or BL (E and F) were first treated with α-AtELP antisera. The bound antibody was visualized with biotinylated goat α-rabbit IgG and then by streptavidin conjugated to 10-nm gold. After a second fixation step and blocking with an excess of BSA, the sections were treated with α-Spo or α-BL antiserum. The bound antibody was visualized with protein A conjugated to 15-nm gold. Large arrows indicate the 15-nm–labeled α-Spo or α-BL antibody and small arrows indicate the 10-nm–labeled α-AtELP antibody in each case. The sections shown in plates C, E, and F reveal colocalization of AtELP and Spo in the Golgi apparatus (G), and structures near the vacuole (V), but no colocalization with BL. The predominant staining of Spo or BL was found in the vacuole (D and F, respectively). Bars, 0.1 μm.

We hypothesize that AtELP serves as a vacuolar sorting receptor in *Arabidopsis* for NTPP-containing proteins. To investigate this possibility in vivo, we performed double-immunogold–labeling studies with transgenic *Arabidopsis* plants expressing the heterologous vacuolar cargo reporter proteins, NTPP-Spo or BL-CTPP. In root sections prepared from transgenic Spo or BL plants, AtELP colocalized with the vacuolar cargo protein NTPP-Spo at the trans-Golgi apparatus (Fig. 3 C), whereas the majority of NTPP-Spo antiserum labeled the vacuole (Fig. 3 D; Table II). However, no colocalization of AtELP and BL-CTPP was observed. Although AtELP and BL-CTPP labeled the same Golgi apparatus, they clearly localized to different parts of the Golgi cisternae, (Fig. 3, E and F). Again, the majority of the BL-CTPP antiserum labeled the vacuole (Fig. 3 F; Table III). Quantitative analysis of the AtELP and Spo colocalization revealed that 74% of the AtELP-labeled gold particles colocalized with 56% of the Spo-labeled gold particles in the Golgi apparatus and structures near the vacuole (Table II). Similar analysis of the AtELP and BL-CTPP localization studies revealed virtually no colocalization of the two proteins in any of the micrographs investigated (Table III). These results, together with those obtained from the in vitro binding assays described above, strongly suggest that AtELP serves as a vacuolar sorting receptor for NTPP-Spo in *Arabidopsis.*

Table II. Relative Distribution of AtELP and NTPP-Spo over Intracellular Compartments in Transgenic Arabidopsis Roots

	Golgi stack	Vacuole	Other structures	Colocalization
AtELP	73	0	27	74
NTPP-Spo	20	70	10	56

Numbers represent the mean percentages of total gold particles found over the indicated compartments over three independent experiments. The percentage of AtELP-specific gold particles that colocalize with the Spo-specific gold particles within each Golgi stack and other structures is also shown. The majority of the labeling for Spo was found in the vacuole. Colocalization was defined as the occurrence of two or more gold particles labeled with AtELP and NTPP-Spo within a distance of 30–50 nm. Approximately 30 independent Golgi apparatuses were examined for each experiment.

Table III. Relative Distribution of AtELP and BL-CTPP over Intracellular Compartments in Transgenic Arabidopsis Roots

	Golgi stack	Vacuole	Other structures	Colocalization
AtELP	82	0	18	0
BL-CTPP	12	80	8	0

Numbers represent the mean percentages of total gold particles found over the indicated compartments over three independent experiments. The percentage of AtELP-specific gold particles that colocalize with the BL-specific gold particles within each Golgi stack and other structures is also shown. The majority of the labeling for BL was found in the vacuole. Colocalization was defined as the occurrence of two or more gold particles labeled with AtELP and NTPP-Spo within a distance of 30–50 nm. Approximately 30 independent Golgi apparatuses were examined for each experiment.

AtELP Interacts with the NTPP of the Endogenous Arabidopsis Vacuolar Protein AtALEU

The vacuolar sorting of NTPP-barley Aleu and NTPP-Spo have been studied in heterologous systems, primarily using tobacco cells (reviewed in Vitale and Raikhel, 1999). As a first step toward understanding the transport of endogenous vacuolar proteins, we sought to identify a vacuolar protein in *Arabidopsis* that could be used to study targeting mechanisms.

The barley cysteine protease Aleu has served as a useful reporter for the study of vacuolar sorting in plants (Holwerda et al., 1992; Holwerda and Rogers, 1993). We cloned a homologue of barley Aleu from *Arabidopsis (AtALEU)* using the barley Aleu amino acid sequence (Rogers et al., 1985) as described in Materials and Methods. At the amino acid level, the predicted ORF encoded by the *Arabidopsis* cDNA was found to be 70% identical to barley Aleu (data not shown). The subcellular location of AtALEU was determined by a combination of organelle fractionation, immunoblot analysis, and electron microscopic immunocytochemistry. Fig. 4 A shows protoplasts prepared from *Arabidopsis* cell suspension culture. Vacuoles were released from protoplasts (Fig. 4 B) and purified by centrifugation on a discontinuous Ficoll gradient. To test the purity of the vacuole preparation, we determined the enzyme activity of two vacuolar-specific enzymes (α-mannosidase and acid phosphatase) in protoplasts and vacuoles. The ratio of enzyme activity of the marker was found to be 20–40-fold higher than in protoplasts (see Fig. 4 C). To investigate the expression of AtALEU for further use as an endogenous vacuolar reporter in *Arabidopsis*, we analyzed total protein extracts from roots and cell suspension by immunoblotting, using affinity-purified α-AtALEU antiserum (Fig. 4 D). The antiserum recognized a band of 29 kD, similar to what was previously found to cross-react with the barley Aleu mAbs when tested against *Arabidopsis* proteins (Rogers et al., 1997). We then examined protoplast and vacuole fractions for the presence of AtALEU by immunoblot analysis, using α-AtALEU antiserum, and with antibodies against marker proteins for the Golgi apparatus (AtELP) and ER (AtSEC12; Fig. 4 E). We found that AtALEU was enriched ~100-fold in the vacuole fraction over the protoplast fraction per microgram of total protein (data not shown). However, the vacuole fraction contained very little AtELP or AtSEC12. To determine the precise subcellular location of AtALEU, we performed immunogold-labeling studies of ultrathin cryosections of *Arabidopsis* roots using the affinity-purified α-AtALEU antiserum. The majority of the α-AtALEU–associated labeling was found in large vacuoles (Fig. 4 E), whereas the preimmune serum showed almost no background labeling (data not shown). To determine whether AtALEU is targeted to the same vacuole as NTPP-Spo, we performed double-immunogold-labeling studies with α-AtALEU and α-Spo antiserum. In root sections prepared from transgenic NTPP-Spo plants, AtLEU colocalized with the vacuolar cargo protein NTPP-Spo in the same large vacuoles (Fig. 4 F). Our results indicate that AtALEU is localized in the same vacuole containing NTPP-Spo and therefore can be used as an endogenous reporter to study vacuolar sorting in *Arabidopsis.*

To investigate the role of AtELP in the transport of endogenous NTPP-containing proteins, we tested its ability to interact with the potential NTPP of AtALEU (Fig. 5 A) by affinity chromatography (see Fig. 1). In these experiments, AtELP was retained on the Wt-NTPP-AtALEU affinity column and was subsequently eluted from the column with the acidic elution buffer (Fig. 5 B). In contrast,

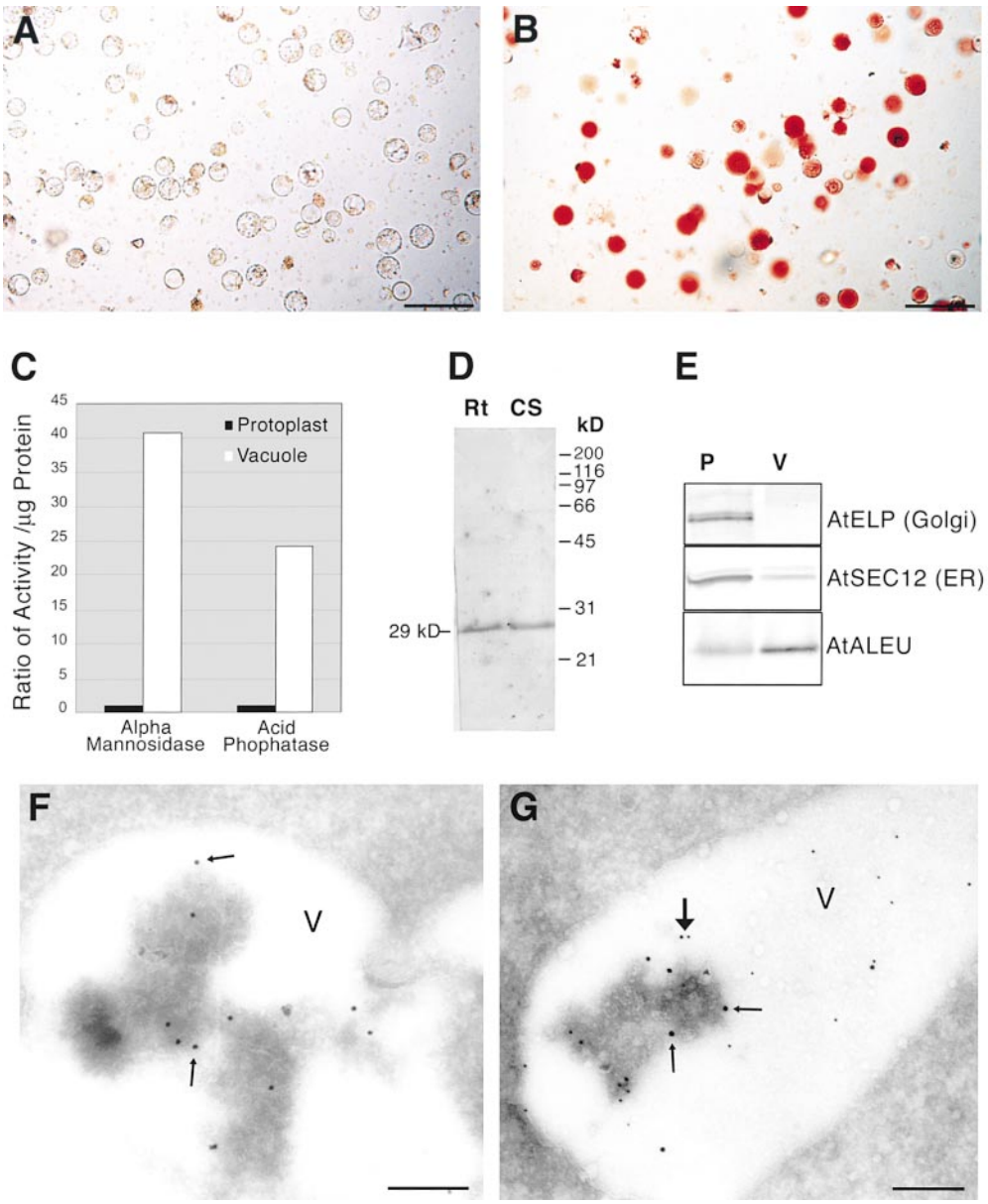

Figure 4. AtALEU is localized in the vacuole in *Arabidopsis.* Vacuoles isolated from protoplasts and examined for the presence of AtALEU. A, Protoplasts were prepared by enzymatic digestion of cell suspension culture. B, Vacuoles stained with neutral red were isolated from protoplasts by centrifugation on a discontinuous Ficoll step gradient. Stained vacuoles were visualized by light microscopy. Bars, 50 μm. C, Graphical representation of the ratio of vacuolar enzyme activities U/μg of protein in purified vacuoles, compared with protoplasts. Enzyme activities of two vacuole-specific enzyme markers were determined in protoplasts from cell suspension culture and vacuoles purified from the same protoplasts. The activities were calculated in mol/liter of methylumbelliferone released per hour per microgram of protein. The ratio of the activity of vacuoles with respect to protoplasts was compared (with protoplasts = 1). D, Immunodetection of AtALEU in root (Rt) and cell suspension culture (CS) indicate the presence of a 29-kD polypeptide recognized by the α-AtALEU antiserum. E, Enrichment of AtALEU in vacuoles. Protein extracts prepared from protoplasts (P) and vacuoles (V) of cell suspension culture (A and B) were analyzed by immunoblotting for the presence of AtALEU, and compared with markers for the Golgi apparatus (AtELP) and ER (AtSEC12). F, Immunogold-labeling of AtALEU in *Arabidopsis* root tissues. Ultrathin cryosections of roots were treated with affinity-purified α-AtALEU antiserum, and the bound antibody was visualized with protein A coupled to 15-nm gold. The arrow indicates the AtALEU-associated 15-nm gold particles. G, AtALEU and Spo are localized in the same vacuoles. In double-immunogold–labeling experiments of AtALEU and Spo, ultrathin cryosections of transgenic *Arabidopsis* roots expressing Spo were first treated with α-AtALEU antisera. The bound antibody was visualized with biotinylated goat α-rabbit IgG and then by streptavidin conjugated to 15-nm gold. After a second fixation step and blocking with an excess of BSA, the section was treated with α-Spo antiserum and the bound antibody was visualized with protein A conjugated to 10-nm colloidal gold. The large arrow indicates the 10-nm–labeled α-Spo antibody and small arrows indicate the 15-nm–labeled α-AtALEU antibody. Bars, 0.1 μm.

no detectable amount of AtELP was retained on the Mt-NTPP-AtALEU column. To determine whether AtELP's interaction with the Wt-NTPP-AtLEU was sequence-specific, we eluted AtELP bound to the Wt-NTPP-AtALEU affinity column at neutral pH by adding 1–1,000 μM of the Wt-NTPP-AtALEU or Mt-NTPP-AtALEU peptides under neutral pH conditions (Fig. 5 C). The Wt-NTPP-AtALEU peptide successfully competed for the binding of AtELP to the corresponding Wt-NTPP-AtALEU peptide column at ~10 μM concentration. The Mt-NTPP-AtALEU peptide, however, did not compete for binding. In addition, the Wt-NTPP-AtALEU peptide competed for binding to the Wt-NTPP-barley Aleu peptide column, suggesting that these peptides compete for the same binding site (Fig. 5 C). Moreover, the patterns of AtELP elution from the Wt-NTPP-barley Aleu affinity column using the Wt-NTPP-AtALEU and the Wt-NTPP-barley Aleu were different. A majority of AtELP bound to the Wt-NTPP-barley Aleu column could be eluted with 100 μM of the Wt-NTPP-AtALEU peptide, with no further elution ob-

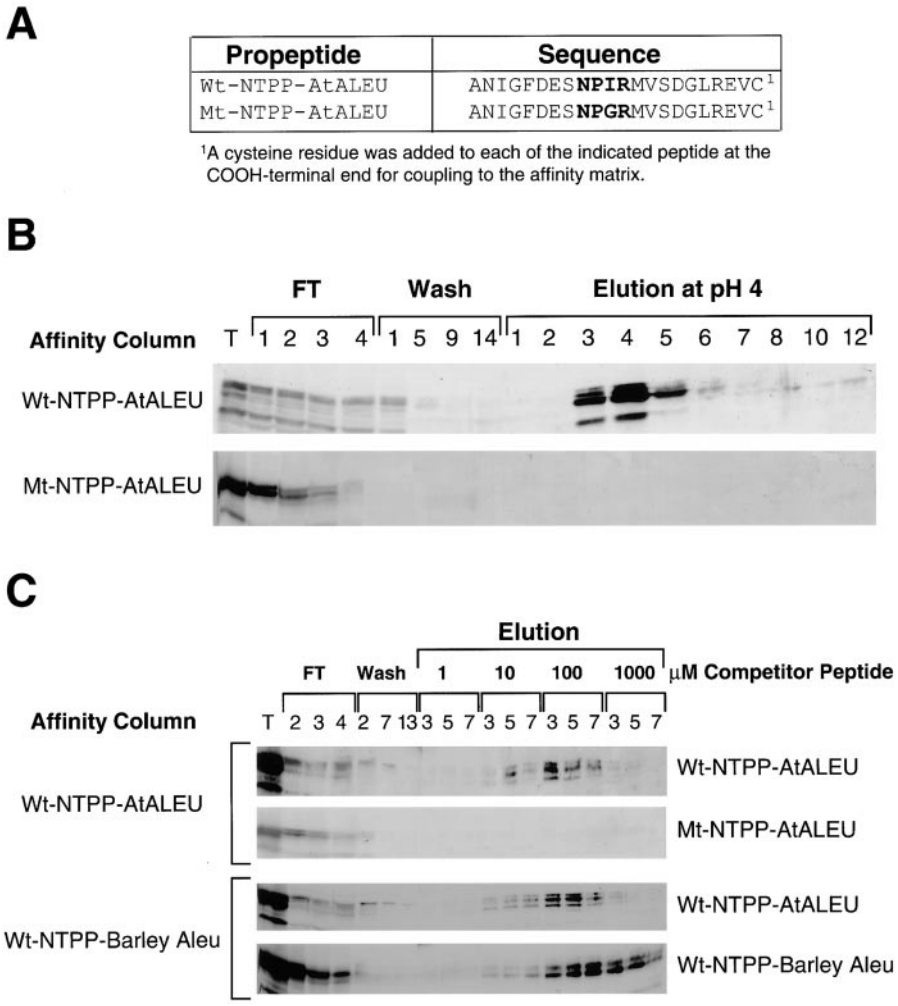

Figure 5. AtELP interacts with the NTPP of AtALEU in a pH-dependent and sequence-specific manner. A, The Wt- and Mt-NTPP-AtALEU peptide sequences used in this experiment are shown. These sequences correspond to the predicted NTPP of AtALEU (Wt-NTPP-AtALEU) or a mutated version of this peptide (Mt-NTPP-AtALEU). B, SDS-PAGE and Western analysis of AtELP binding to the Wt-NTPP– and Mt-NTPP–targeting propeptides of AtALEU. Binding assay with peptide affinity columns were identical to that used in Fig. 1 A. C, Binding of AtELP to the NTPP-AtALEU propeptide is sequence-specific. The specificity of AtELP binding to the Wt-NTPP-AtALEU propeptide was determined in a competition assay identical to that described in Fig. 1 B using increasing concentrations (1–1,000 μM) of the indicated peptides as competitors in the same buffer to elute AtELP bound to the Wt-NTPP-AtALEU peptide column. The Wt-NTPP-barley Aleu and Wt-NTPP-AtALEU peptides bind to the same site on AtELP. AtELP bound to the Wt-NTPP-barley Aleu peptide column can be eluted with increasing concentration of the Wt-NTPP-AtALEU peptide in the same buffer at pH 7 in the competition assay. For each of the flow through (FT), wash, and elution steps, fractions were collected, and the indicated fraction numbers were analyzed by immunoblotting with α-AtELP antiserum as in Fig. 1 B.

served at 1,000 μM. Although a significant amount of AtELP bound to the same affinity column could be eluted with similar concentration (100 μM) of the Wt-NTPP-barley Aleu peptide, a tenfold higher concentration (1,000 μM) of the Wt-NTPP-barley Aleu peptide was required to elute completely the additional AtELP that remained bound to the column. Thus, AtELP may have a higher affinity for the putative NTPP-sorting signal of the endogenous AtALEU from *Arabidopsis* than the signal present on barley Aleu. We considered these results in conjunction with our other findings that: AtELP interacts with various NTPPs, but not CTPPs; and it colocalizes with NTPP-Spo, but not with BL-CTPP in transgenic *Arabidopsis*. We propose that AtELP is a vacuolar sorting receptor involved in the transport of NTPP-containing proteins to the vacuole in *Arabidopsis*.

Discussion

Compared with our understanding of the molecular machinery involved in vacuolar/lysosomal protein targeting in yeast and mammalian cells, we know very little about the plant sorting machinery. Evidence suggests that the vacuolar transport processes in plants may be more complex than their counterparts in yeast or mammalian cells. Both biochemical and microscopy data indicate that there are at least two vesicle-mediated transport pathways for the targeting of soluble proteins to two functionally different vacuoles in plants (reviewed in Vitale and Raikhel, 1999). More recent evidence has revealed the presence of intermediate compartments (e.g., the PVC) for each of the pathways (Conceição et al., 1997; Paris et al., 1997; Robinson et al., 1998).

We have characterized a sorting receptor-like protein, AtELP, from *Arabidopsis* whose biochemical properties and subcellular location suggest that it may be a vacuolar sorting receptor (Ahmed et al., 1997; Sanderfoot et al., 1998). In this report, we show that AtELP specifically interacts with the NTPP-sorting signals of the plant vacuolar proteins, Spo and barley Aleu, but not with the CTPP-sorting signals of BL or TobChit. Moreover, we have isolated and characterized an endogenous NTPP-containing vacuolar protein from *Arabidopsis*, AtALEU, whose potential NTPP was also found to interact with AtELP, in a pH-dependent, sequence-specific manner. The pH level along the vacuolar-transport pathway is known to decrease (reviewed in Neuhaus and Rogers, 1998). The low pH in the late organelle(s), such as a PVC or vacuole, appears to be important for sorting, because treatment of plant cells with ionophores (for example, monensin) or

v-ATPase inhibitors causes missorting of vacuolar precursor proteins, including Spo (Matsuoka et al., 1995; Matsuoka et al., 1997). Thus, any potential binding of AtELP to the vacuolar sorting signals would occur at the Golgi apparatus with a neutral (or slightly acidic) condition and the interaction would be disrupted at the more acidic pH condition in the PVC or vacuole, as observed in the case of the mannose-6-phosphate receptor (M-6-PR) interaction with the M-6-P sorting signal in mammalian cells (reviewed in Dahms et al., 1989). Consistent with this, we have found that AtELP interacts with the NTPPs of sweet potato Spo and barley Aleu, and with the putative NTPP-sorting signal of the endogenous *Arabidopsis* vacuolar protein AtALEU at neutral pH; the bound receptor can be eluted under acidic elution conditions.

In our competition experiments, the Wt-NTPP-barley Aleu peptide competed ~10-fold more strongly than the Wt-NTPP-Spo peptide for binding to AtELP. Similar observations were made for another potential sorting receptor, BP-80, isolated from pea (Kirsch et al., 1994). This differential competition for binding to AtELP could be attributed to the differences in the binding motifs present in the two NTPP-sorting signals tested. Although both NTPPs contain the characteristic NPIR motif, which is required for the binding of AtELP, the in vivo functional importance of this motif has been demonstrated only for Spo (Matsuoka and Nakamura, 1991). In addition, recent analysis of the NTPP of Spo precursor indicates that the amino acid requirement to function as a sorting signal within the NPIRL motif is X_1-X_2-I/L-X_3-X_4, where Asn (N) is the preferred residue. Residues at position X_1 and X_2 may not be an acidic amino acid, X_3 may be any residue, and X_4 must be a large and preferably hydrophobic residue (Matsuoka and Nakamura, 1999). Moreover, NTPP-Spo contains the sequence NPIRL (see Fig. 1 A), where X_4 is a hydrophobic Leu residue. In contrast, the efficient vacuolar targeting of barley Aleu requires the presence of three separate contiguous determinants within the Wt-NTPP-barley Aleu sorting signal, one of which contains the NPIR motif (Holwerda et al., 1992). The NPIR motif alone as the sorting signal is capable of targeting only 3–7% of the protein to the vacuole. Therefore, sequences surrounding this motif in NTPP-barley Aleu may play a role in its interactions with the sorting receptor. Whether these surrounding sequences interact with the receptor in a mechanism that is dependent on the NPIR motif is unknown. There may be different domains within AtELP that are capable of differentially interacting with multiple signals. In this regard, Vps10p, a sorting receptor for the yeast carboxypeptidase Y (CPY), has been shown to contain multiple sites for binding several vacuolar proteins (Jorgensen et al., 1999). Alternatively, because there appears to be multiple isoforms or homologues of AtELP in *Arabidopsis* (as well as of BP-80 in pea), the various NTPPs tested in this report may interact with each of the isoforms/homologues of AtELP with different affinities. Additionally, the isoforms may have developmental or tissue-specific expression. Thus, it would be interesting to understand the functional interactions between the variations of the NTPP-sorting signals and the several isoforms or homologues of AtELP in targeting vacuolar proteins.

Our binding studies show that the well-characterized CTPPs of BL (Wt-BL-CTPP) or TobChit (Wt-TobChit-CTPP) do not bind any detectable level of AtELP. Moreover, no cross-reacting proteins bind to either of the CTPP peptides, suggesting that proteins with a CTPP must use a different cargo receptor. We have been unable to identify any common motif among the CTPP signals identified thus far; it is possible that a common secondary structure present within the CTPPs serves as the sorting determinant recognized by a potential receptor. This potential receptor would most likely be involved in the transport of CTPP-containing proteins by a pathway different from that used by AtELP and NTPP-containing proteins, as it has been demonstrated that wortmannin inhibits selectively the targeting of BL-CTPP, but not of NTPP-Spo in tobacco cells (Matsuoka et al., 1995). However, more direct evidence of protein sorting through these two pathways has been lacking. The experimental evidence presented here provides the first direct demonstration of the specific colocalization of a plant vacuolar sorting receptor (AtELP) with a vacuolar cargo protein containing an NTPP-sorting signal (NTPP-Spo), but not with a CTPP-containing vacuolar protein (BL-CTPP) on the same Golgi cisternae, and in structures near the vacuole, that could represent the PVC. In these *Arabidopsis* root cells, of the organelles located close to the Golgi apparatus or vacuole that were labeled with AtELP, nearly half were also labeled with NTPP-Spo. In contrast, very little if any AtELP was observed to colocalize with BL-CTPP in similar experiments. The preferential interaction of AtELP with the NTPP-sorting signals and its high degree of colocalization with NTPP-Spo indicate that NTPP-containing proteins destined to the LV are likely sorted by AtELP. In this regard, we have isolated and characterized an endogenous NTPP-containing vacuolar protein from *Arabidopsis*, AtALEU, whose potential NTPP was also found to interact with AtELP, in both a pH-dependent and sequence-specific manner. The receptor's localization at the TGN, the PVC, and in CCVs suggests that these organelles are involved in the transport of vacuolar proteins (Sanderfoot et al., 1998). In addition, AtELP was previously found to colocalize with several components of the vesicle transport machinery at the TGN, characterized in *Arabidopsis* (Bassham and Raikhel, 1998; Zheng et al., 1999). Further, the biochemical characteristics of AtELP, together with the preferential interaction of its cytoplasmic tail with the TGN-associated AP-1 clathrin-adaptor complex, are consistent with the selective function of the protein in the TGN with subsequent sequestration of the receptor-cargo complex into CCVs. These results strongly suggest that AtELP, together with NTPP-Spo, is sorted out of the TGN via AP-1–containing CCVs.

The evidence presented in this paper indicates a role for AtELP in the sorting of NTPP-containing proteins to the vacuole in *Arabidopsis*. However, the nature of its endogenous cargo remains largely unknown, primarily because very few endogenous soluble vacuolar proteins have been characterized in this plant. Characterization has been difficult because many vacuolar proteins, or enzymatic activities associated with them, are found in both vacuolar and secreted forms (Vitale and Raikhel, 1999). The isolation and characterization of AtALEU reported in this study may now help us to investigate the in vivo role of AtELP

and many other components of the transport machinery identified thus far in *Arabidopsis* (reviewed in Vitale and Raikhel, 1999), in the transport of NTPP-containing vacuolar proteins in this otherwise model plant system. Future work directed toward understanding the in vivo nature of the interactions between AtELP and its related family of vacuolar sorting receptors with the endogenous vacuolar proteins in *Arabidopsis* is likely to reveal more important information regarding the complex nature of the vacuolar transport pathways in plants.

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