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CHARACTERIZATION AND FUNCTIONAL ANALYSIS OF A VACUOLAR PROTEIN SORTING RECEPTOR IN ARABIDOPSIS

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CHARACTERIZATION AND FUNCTIONAL ANALYSIS OF A VACUOLAR PROTEIN SORTING RECEPTOR IN ARABIDOPSIS

By

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ABSTRACT

CHARACTERIZATION AND FUNCTIONAL ANALYSIS OF A VACUOLAR PROTEIN SORTING RECEPTOR IN ARABIDOPSIS

By

Sharif Uddin Ahmed

The plant vacuole is a multifunctional organelle essential for numerous functions vital to plant cell survival. The proper maintenance of its identity and function is dependent on the appropriate targeting of various membrane and soluble proteins to the organelle. Many soluble proteins destined for the plant vacuole are initially synthesized at the endoplasmic reticulum from where they travel through the Golgi complex. Upon reaching the *trans*-Golgi network (TGN), vacuolar proteins are sorted away from proteins destined for secretion. This sorting step requires the presence of positive signals on the vacuolar proteins that are recognized by transmembrane cargo receptors at the TGN, that direct some of these proteins into clathrin-coated transport vesicles (CCVs), destined for the vacuole. Two different types of sorting signals have been characterized in detail for soluble vacuolar proteins in plants. Some proteins contain a cleavable NH_2-terminal propeptide (NTPP) that function as a targeting determinant; others contain a cleavable COOH-terminal propeptide (CTPP). It has been previously shown that the NTPP- and CTPP-mediated sorting occurs by biochemically distinct pathways. In addition, targeting of soluble proteins to plant vacuole has been found to be saturable, indicating the involvement of sorting receptors. Although the sorting signals are well characterized, much less is known about the proteins that interact with these sorting signals and the identity of the vesicles that carry proteins from the TGN to the vacuole in plant cells.

Towards understanding the molecular mechanisms of protein sorting to the plant vacuole, I have isolated a protein from Arabidopsis, AtELP (*Arabidopsis thaliana* epidermal growth factor receptor-like protein), which shares all the features common to eukaryotic protein sorting receptors. Biochemical, subcellular fractionation and immuno-electron microscopy studies indicate that, AtELP is a type I integral membrane protein present in the Golgi apparatus, in CCVs and in an intermediate prevacuolar compartment, containing the marker protein, AtPEP12p. Further, the AtELP cytoplasmic tail preferentially interacts with the mammalian TGN-specific, clathrin-adaptor protein complex, suggesting a likely role of AtELP in CCV-directed trafficking at the TGN.

The potential involvement of AtELP in plant vacuolar targeting was analyzed by examining its ability to interact with various vacuolar sorting signals, using an *in vitro* binding assay. These experiments reveal that AtELP binds to the NTPPs of barley pro-aleurain and sweet potato pro-sporamin, in a pH dependent and sequence-specific manner. The putative receptor does not bind to the CTPPs of pro-barley lectin or pro-tobacco chitinase. The binding of AtELP to the aleurain and sporamin NTPP sorting signals is dependent on the "NPIR" targeting motif previously found to be important for proper *in vivo* sorting of these cargo proteins to the plant vacuole. These biochemical results are supported by electron microscopy studies, which reveal the colocalization of AtELP with the vacuolar cargo protein sporamin at the Golgi apparatus and structures near the vacuole, but not with the barley lectin cargo protein in roots of transgenic *Arabidopsis* plants. Taken together, all of these results strongly suggest that AtELP functions as a vacuolar sorting receptor involved in the targeting of NTPP-containing proteins in *Arabidopsis*.

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ER	= Endoplasmic reticulum
TGN	= *trans*-Golgi network
CCV	= Clathrin-coated vesicle
NTPP	= NH_2-terminal pro-peptide
CTPP	= COOH-terminal pro-peptide
EGF	= Epidermal growth factor
EGFR	= Epidermal growth factor receptor
At	= Arabidopsis thaliana
AtELP	= Arabidopsis thaliana EGFR-like protein
PVC	= Pre-vacuolar compartment
NSF	= N-ethylmaleimide-sensitive factor
SNAP	= Soluble NSF attachment protein
SNARE	= SNAP receptors
t-SNARE	= target-SNARE
v-SNARE	= vesicle SNARE
AP	= Adaptor protein
Spo	= Sporamin
BL	= Barley lectin
Aleu	= Aleurain
TIP	= Tonoplast intrinsic protein
Vps	= Vacuolar protein sorting
SDV	= Smooth dense vesicles
PAC	= Precursor accumulating vesicles
LV	= Lytic vacuole
PSV	= Protein storage vacuole
BiP	= Binding protein
BP-80	= Binding protein of 80 kD
TLG	= t-SNARE at late Golgi
CPY	= Carboxy peptidase Y
PEP	= Peptidase deficient
ALP	= Alkaline Phosphatase
VTI	= Vps10-interacting factor
VAM	= Vacuolar morphology
PM	= Plasma membrane
M-6-P	= Mannose-6-phosphate
M-6-PR	= Mannose-6-phosphate receptor
EST	= Expressed sequence tag
LDL	= Low density lipoprotein
LDLR	= Low density lipoprotein receptor
GST	= Glutathione-S-transferase
ERD	= Endoplasmic reticulum-retention defective

SAR	= Secretion-associated and ras-superfamily-related
SEC	= Secretion defective
CHC	= Clathrin heavy chain
LAMP	= Lysosome associated membrane protein
VSS	= Vacuolar sorting signal
VSR	= Vacuolar sorting receptor
ARA	= Arabidopsis ras -related
TobChit	= Tobacco Chitinase
ORF	= Open reading frame

Chapter 1

Introduction

An Overview of Protein Transport to the Plant Vacuole

I. Plant Vacuole

The endomembrane system of plant cells consists of distinct membrane bound organelles, of which the vacuole is the largest. The plant vacuole performs a diverse set of functions, many of which are essential for the regulation and maintenance of plant growth and development (reviewed in Marty, 1999). Unlike yeast and mammalian vacuoles/lysosomes, the plant vacuole may serve as both a lytic compartment for the degradation of material and a storage organelle. For example, vacuoles serve as reservoirs for ions and secondary metabolites such as alkaloids, and glycosides, organic acids, and pigments, and thus play a crucial role in the process of detoxification and general cell homeostasis in plants. They may also store reserve proteins and soluble carbohydrates. On the other hand, vacuoles also contain many hydrolytic enzymes that are used to hydrolyze some of the storage proteins, when necessary. In this regard, vacuoles are analogous to the lysosomes of animal cells. Additionally, they act in combination with the cell wall to generate turgor, which is essential for the maintenance of cell shape and size.

In most cells from vegetative tissues, the central vacuole occupies a majority of the cell volume and is essential for much of the physiology of the plant. However, in some specialized plant cells, multiple vacuoles are found that are different in size, shape and most notably in content and function. In recent years, various molecular, biochemical and microscopy techniques have been used to characterize specialized vacuolar compartments from different plant tissues (Hoh et al., 1995, Paris et al., 1996; Swanson et al., 1998). These recent studies provide evidence suggesting that distinct vacuoles may simultaneously function in the same plant cell. At least two different vacuoles, one

corresponding to a lytic vacuole (LV), and another representing a protein storage vacuole (PSV), have been clearly identified (Paris et al., 1996; Swanson et al., 1998, Jiang and Rogers, 1998; 1999; Di Sansebastiano et al., 1998). As for many other intracellular compartments, the proper identity and function of these different vacuoles is maintained by the appropriate targeting and transport of various membrane and soluble proteins. These proteins often serve as markers for each type of vacuole. For example, the membrane, or tonoplast, of many vacuoles contain specific marker proteins known as tonoplast intrinsic proteins (TIPs), which have been suggested to define the identity of each type of vacuole (Neuhaus and Rogers, 1998). Similarly, different types of soluble proteins are found to be transported to functionally different vacuoles. Significant progress has been made in the last decade in understanding the molecular mechanisms involved in the transport of proteins to the plant vacuole. The following sections summarize the current information available on many of the molecular aspects of the plant vacuole protein sorting machinery.

II. Plant Vacuolar Sorting Signals

Many soluble and membrane proteins destined for the plant vacuole are initially synthesized at the endoplasmic reticulum (ER) from where they travel through the Golgi complex. Upon reaching the trans-Golgi network (TGN), vacuolar proteins are sorted away from proteins destined for secretion (reviewed in Beevers and Raikhel, 1998; Vitale and Raikhel, 1999). This sorting step requires the presence of positive signals on the vacuolar proteins that work as a "molecular tag" for vacuolar delivery. In the absence of such information tags, these proteins are secreted to the extracellular space. Addition of these signals to otherwise secreted proteins can redirect the chimeric protein to the

vacuole. Three different types of sorting signals have been described for soluble vacuolar proteins in plants (Chrispeels and Raikhel, 1992). Some proteins, such as barley aleurain and sweet potato sporamin, contain a cleavable NH_2-terminal propeptide (NTPP) that functions as a targeting determinant; others (e.g. barley lectin, tobacco chitinase, phaseolin, and Brazil nut 2S albumin) contain a cleavable COOH-terminal propeptide (CTPP). Finally, some proteins, such as phytohemagglutinin and legumin, contain an internal targeting determinant that is part of the mature protein (Figure 1.1).

II-a. NTPP Signals

The NTTP signals contain an NPIR consensus amino acid motif which does not tolerate much alterations (reviewed in Neuhaus and Rogers, 1998; Matsuoka and Neuhaus, 1999). This motif is necessary and sufficient for the targeting of prosporamin to the vacuole (Nakamura and Matsuoka, 1993; Matsuoka et al., 1995). In the cysteine protease proaleurain, separate contiguous determinants in the NTPP, one of which contains the NPIR motif, contribute to the maximum level of vacuolar transport of aleurain (Figure 1.2). Although the NPIR motif is critical for the NTPP's function, a strict conservation of these amino acids may not be essential to constitute the vacuolar sorting signal. Detailed mutational analysis of this region in sporamin has recently demonstrated that the large and hydrophobic alkyl chain of the third position (I) is essential (Matsuoka and Nakamura, 1999). This isoleucine can only be changed to leucine without losing the function of the vacuolar sorting signal. Moreover, the NTPP of the prosporamin can also function as a vacuolar sorting signal when located at the COOH-terminus of the mature protein (Koide et al., 1997). This suggests that there is no obvious need for the sequence-specific signals to be included within an NH_2-terminal propeptide. Sporamin is

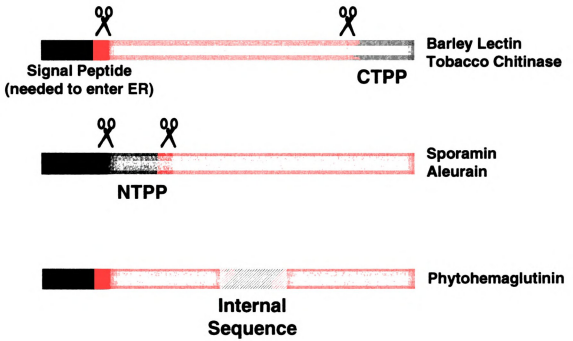

Figure 1.1
Three classes of vacuolar targeting determinants identified in plants. NTPP, NH_2-terminal propeptide; CTPP, COOH-terminal propeptide; Internal Sequence, targeting signal that is part of the mature protein.

NTPPs

Sweet Potato Sporamin	**HSRFNPIRLPTTHEPA**
Barley Aleurain	**SSSSFADSNPIRPVTDRAAS**
Rice Oryzains	**ASSGFDDSNPIRSVTDHAA**

CTPPs

Barley Lectin	**VFAEAIAANSTLVAE**
Tobacco Chitinase	**GLLVDTM**
Brazil Nut 2S Albumin	**IAGF**
Bean Phaseolin	**AFVY**

Figure 1.2
Sequence comparison of representative propeptides that function as vacuolar targeting signals.
NTPPs, NH_2-terminal propeptides; CTPPs, COOH-terminal propeptides (reviewed in Vitale and Raikhel, 1999; Matsuoka and Neuhaus, 1999)

transported to the large vacuoles in cells from sweet potato tuber roots (Maeshima et al., 1985), whereas aleurain is delivered to a lytic vacuole (LV) distinct from protein storage vacuoles (PSVs) in pea root tip cells (Paris et al., 1996).

II-b. CTPP Signals

In contrast to the NTPP signals, no consensus sequence has yet been identified for the CTPP targeting domains (Figure 1.2). These are often enriched in hydrophobic amino acids, but the importance of the hydrophobic nature is not absolute (reviewed in Matsuoka and Neuhaus, 1999). CTPPs from vacuolar proteins differ in length, anywhere from less than 7 amino acid-long to over 30 residues. They can be as short as only three terminal amino acids, as indicated by the predominant vacuolar localization of an altered form of barley lectin containing only three residues at the COOH-terminus (Dombrowski et al., 1993). Similarly, in an altered form of tobacco chitinase A, six residues were sufficient, many of which could be replaced with random sequences, and still function as vacuolar sorting signals (Neuhaus et al., 1994). In both cases, the function of these vacuolar sorting signals could be hindered by the addition of one or more Gly residues or sugar modifications to the end of the propeptide (Dombrowski et al., 1993; Neuhaus et al., 1994). These data suggest that rather than sequence specificity, perhaps a common structural feature may serve as the sorting signal in the CTTPs. Recent results indicate that more than one sorting mechanism may exist for the transport of CTPP-containing proteins from the Golgi to the vacuole in plants, and that the CTPP- and NTPP-dependent pathways are biochemically distinct (Frigerio et al., 1998; Matsuoka et al., 1995).

II-c. Internal Signals

Other plant vacuolar proteins are synthesized without a cleavable vacuolar sorting

signal. Targeting information for the bean phytohemagglutinin and vicilin is present within the mature portion of the proteins, both of which are delivered to the PSVs (Tague et al., 1990; Saalbach et al., 1991). However, the sequence requirements and structural features of this type of signal is not very well characterized.

III. Targeting Pathways to the Plant Vacuole

The use of several different experimental approaches have established the presence of multiple protein transport pathways to the vacuole. There appears to be at least two, or may be three, major sorting mechanisms to target proteins to perhaps as many as three types of vacuoles. A working model of the routes of soluble proteins to the different vacuoles is shown in Figure 1.3. It is now known that proteins containing sequence specific vacuolar sorting signals, such as NTPPs, are transported from the Golgi to the LV in clathrin-coated vesicles (CCVs). Other proteins are directed to PSVs by either Golgi-dependent or Golgi-independent pathways, depending on whether aggregation of these proteins occurs in the ER or later. For example, some seed storage proteins (such as vicilin, legumin) containing internal signals accumulate in non-clathrin coated dense vesicles (DVs) that bud off from the *trans*-Golgi, and ultimately accumulate in PSVs (Robinson et al., 1998; Vitale and Raikhel, 1999). Other storage proteins (such as globulin and albumin in pumpkin seeds) are transported from the ER to the PSV via large precursor-accumulating vesicles (PACs), which are distinct from Golgi-derived vesicles but similar to the late protein bodies described in pea cotyledons (Hara-Nishimura et al., 1998). In bean seeds however, Golgi-processed storage glycoproteins are incorporated into ER-derived PACs at the periphery of the core aggregates. These storage glycoproteins, together with other storage proteins, are ultimately packaged into the

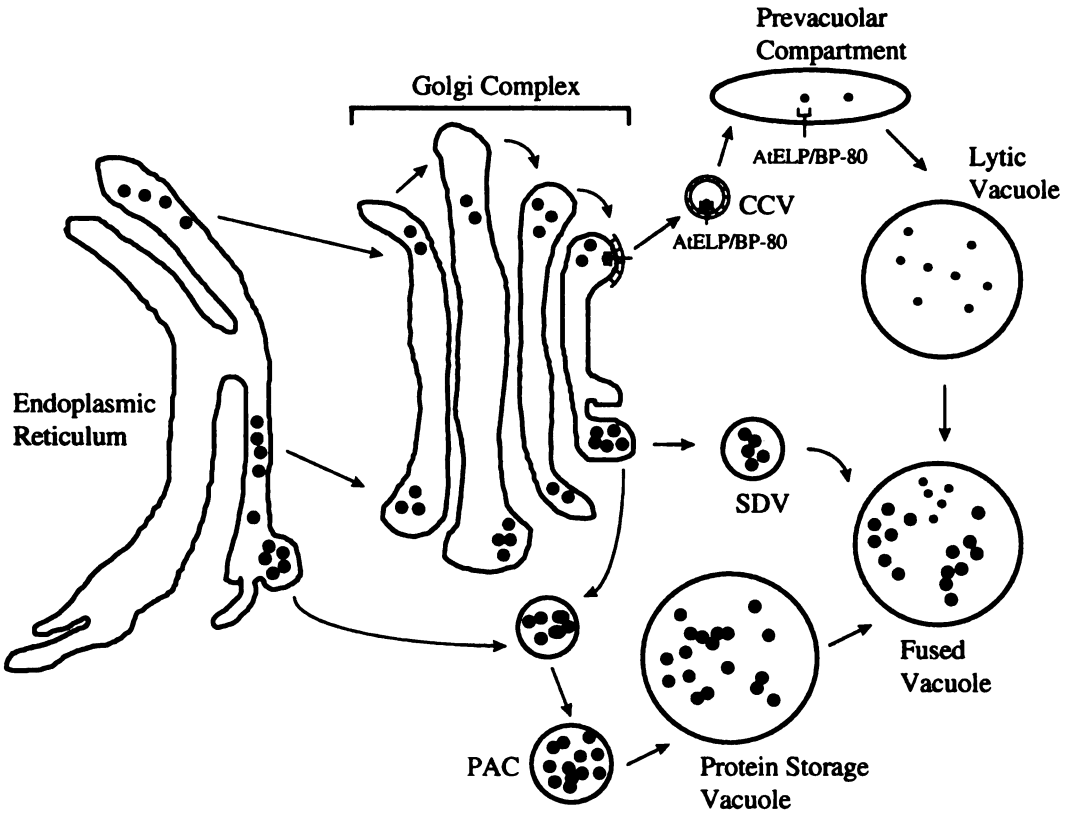

Figure 1.3
A working model for the different transport pathways used by soluble vacuolar proteins to the different vacuoles. The red dots indicate a protein targeted to lytic vacuoles by clathrin-coated vesicles (CCVs), via a prevaucolar compartment. The transport of some of these proteins may be mediated by the AtELP/BP-80 family of vacuolar sorting receptor. The blue and black dots indicate two proteins directed to protein storage vacuoles. These proteins may be transported either through the Golgi-apparatus or follow a Golgi-independent pathway, depending on whether they form aggregates at the endoplasmic reticulum or later. These two different routes could be mediated by smooth dense vesicles (SDVs) or by precursor accumulating vesicles (PACs). The fused vacuole represents the situation in many mature cells, where a central vacuole is created from the fusion of lytic and a protein storage vacuoles. (Adapted from Vitale and Raikhel, 1999) into ER-derived PACs at the periphery of the core aggregates.

mature vesicles for transport to the PSV (Marty, 1998). A third possible pathway appears to transport CTPP-containing proteins to a vacuole, distinct from the lytic vacuole (Paris et al., 1996). This pathway appears to be biochemically different from the NTPP-mediated transport pathway (Matsuoka et al., 1995). Although some progress has been made in recent years in understanding the transport pathways at the molecular level, it is still unclear as to whether the two or three transport pathways identified so far, lead to two or three functionally different vacuoles. This is further complicated by the possibility of the different small vacuoles fusing with each other to form a large central vacuole that is capable of receiving both lytic and storage proteins. This potential fusion mechanism may explain as to why the NTPP-containing sporamin was found together with a CTPP-containing BL in the same large central vacuole in transgenic tobacco plants (Schroeder et al., 1993)

The complex nature of the plant vacuolar protein transport machinery is even more evident when the transport of vacuolar integral membrane proteins is considered. Soluble and integral membrane vacuolar proteins have been shown to be targeted to the vacuole by different mechanisms. For example, pharmacological studies have revealed that newly synthesized vacuolar membrane protein α-TIP and the soluble vacuolar protein PHA arrive at the same vacuolar destination by traveling through different routes (Gomez and Chrispeels, 1993). Moreover, integral membrane protein sorting to vacuoles in plant cells have been shown to involve at least two different pathways (Jiang and Rogers, 1998). These include a direct ER to PSV pathway, and a separate pathway via the Golgi to the LV.

IV. Components of the Vacuolar Sorting Machinery

IV-a. *Vacuolar Protein Sorting Receptors*

Several potential components of the plant vacuolar transport machinery have been identified recently. A potential vacuolar sorting receptor that may be involved in the NTPP-mediated transport to the LV, has been isolated and characterized at the molecular level by two independent methods (Kirsch et al; 1994; Ahmed et al., 1997; Paris et al; 1997). The protein is an ~80 kD type I integral membrane glycoprotein that was first purified from pea CCVs, based on its ability to interact with the NTPP targeting determinants of barley aleurain and sporamin (hence named BP-80). BP-80 was found to bind the aleurain and sporamin NTPPs at neutral pH and dissociate from it in acidic pH conditions (Kirsch et al., 1994). In addition, BP-80 was found to interact with the CTPP of Brazil nut 2S albumin (Kirsch et al., 1996), and more recently, a protein immonologically related to BP-80 was found to associate with a proteinase inhibitor containing a potential CTPP, in tobacco cells (Miller et al., 1999). Some of the binding properties of BP-80 were found to be consistent with a possible role for the protein in sorting vacuolar proteins at the Golgi, for eventual delivery to the vacuole.

Using a genomic approach, I have independently isolated and characterized an ~ 80kD type I integral membrane protein from *Arabidopsis thaliana*, that contains several features common to many eukaryotic protein sorting receptors (Chapter 2 and 3). Primarily, AtELP is similar to the mammalian epidermal growth factor receptor (hence named AtELP, for *Arabidopsis thaliana* EGF receptor-like protein). AtELP and BP-80 are >70% identical to each other at the amino acid level. AtELP is enriched in plant CCVs, and it is localized at the *trans*-Golgi Network (TGN), and in a prevacuolar

compartment. Further, the AtELP cytoplasmic tail can preferentially interact with the mammalian TGN-specific, clathrin-associated AP-1 complex of proteins *in vitro* (Chapter 2 and 3). Similarly, BP-80 is present at the dilated ends of the Golgi and in prevacuoles capable of fusing with the vacuole (Paris et al., 1997). Most recently, we have found that AtELP also interacts with several different NTPP-targeting determinants, and that in transgenic Arabidopsis roots, AtELP colocalizes with the NTPP-containing protein, sporamin, but not with the CTPP-containing protein, BL (Chapter 4). An interesting observation regarding the genomic organization of AtELP/BP-80 is that they are both members of a multi-gene family. Four BP-80-related cDNAs have been identified from pea (Paris et al., 1997), while there appears to be at least five AtELP-related cDNA and genomic clones in Arabidopsis (Chapter 5). Perhaps the multiple forms of AtELP/BP-80 may function in the same pathway and each may either have developmental or tissue-specific expression, or even different affinity for slightly different versions of the NTPP-sorting signals. Alternatively, the different AtELP/BP-80 family members may interact with the other vacuolar sorting signals identified in plants.

IV-b. Vesicle Transport Components

Several other candidate genes have been isolated from *Arabidopsis thaliana* that are thought to be involved in plant vacuolar targeting. These appear to encode protein components of the vesicle transport machinery that may be involved in the transport of vacuolar cargo proteins from the TGN to a prevacuolar compartment (PVC). The characteristics and subcellular location of the potential components of the transport machinery identified so far are listed in Table 1 together with their predicted function. In particular, these proteins may participate in the vesicle docking and fusion process, which

is regulated by a set of membrane receptors termed SNAREs (for soluble N-ethylmaleimide-sensitive factor attachment protein receptors) (reviewed in Sanderfoot and Raikhel, 1998). According to the existing model, SNAREs on vesicles (v-SNAREs) interact with their cognate SNAREs on the target membrane (t-SNAREs). This interacting complex is further bound by several soluble regulatory factors, eventually leading to membrane fusion. The docking and fusion steps are further regulated by additional factors that include small GTPases and Sec1-related proteins.

In yeast, docking and fusion of Golgi-derived vesicles with the PVC is directed by the interactions between v-SNARE, Vti1p, and the t-SNARE, Pep12p, as well as the Sec1p-related protein Vps45p (Fischer von-Mollard et al., 1997). An additional t-SNARE, Vam3p, is required for transport from the PVC to the vacuole (Wada et al., 1997), as well as other transport steps (Darsow et al., 1997; Ungermann et al., 1999). Over the last several years functional complements of the yeast t-SNAREs Pep12p and Vam3p, AtPEP12p and AtVAM3p have been characterized, respectively from Arabidopsis (Bassham et al., 1995; Conceição et al., 1997; Sato et al., 1997; Sanderfoot et al., 1999). These have been isolated by complementation of yeast mutants, defective in each of the respective genes, with Arabidopsis cDNAs. Although, their subcellular distribution is consistent with their predicted function in vacuolar targeting, there appears to be several important differences between the plant t-SNAREs and their yeast counterparts, that may reveal functional differences. In yeast, Pep12p is localized to the pre-vacuolar endosome and its activity is required for transport of proteins from the Golgi to the vacuole through a well defined route, the carboxypeptidase Y (CPY) pathway (Becherer et al., 1996). Further, the recycling of the CPY sorting receptor, Vps10p, between the late-Golgi and

Protein	Biochemical Properties	Subcellular Location	Predicted Function
AtELP/BP-80	Type I Integral membrane protein	TGN, CCVs, PVC	Vacuolar Sorting Receptor involved in TGN to PVC transport
AtPEP12p	Type II Integral membrane protein	PVC	t-SNARE interacting with AtVTI1a during membrane fusion
AtVAM3	Type II Integral membrane protein	PVC	t-SNARE interacting with AtVTI1a during membrane fusion
AtVTI1a	Type II Integral membrane protein	TGN, PVC	v-SNARE interacting with AtPEP12p during membrane fusion
AtTLG2a	Type II Integral membrane protein	TGN	t-SNARE interacting with AtVPS45p during membrane fusion
AtVPS45p	Peripheral membrane	TGN	Sec-1 homolog interacting with AtTLG2a during membrane fusion

Table 1.
Potential components of the plant vacuolar sorting machinery identified thus far. The biochemical properties, subcellular location and predicted function of known plant proteins thought to be involved in vacuolar targeting. For references, please see text.

PVCs, is essential for the receptor's function (Cooper and Stevens, 1996). On the other hand, Vam3p is localized to the vacuole (Wada et al., 1997) where it mediates delivery of cargo from both the CPY and the recently described alkaline phosphatase (ALP) pathways (Darsow et al., 1998). Similar to the yeast t-SNARE, Pep12p, AtPEP12p is located on a PVC in Arabidopsis roots (Conceição et al., 1997), and it forms a characteristic 20S SNARE complex, *in vivo* (Bassham and Raikhel, 1999). In addition, AtPEP12p and AtELP colocalize on a PVC (Chapter 3), suggesting that the PVC serves to recycle plant vacuolar cargo receptors back to the TGN. However, AtVAM3p appears to have a cell-type-specific location in Arabidopsis. In roots, AtVAM3p is located on the PVC (Sanderfoot et al., 1999), while in undifferentiated meristematic cells, it is found on the vacuole (Sato et al., 1997). This suggests that AtVAM3p may have cell-specific functions in Arabidopsis.

Functional equivalents of the yeast v-SNARE Vti1p, AtVTI1a and b, have also been characterized in *Arabidopsis* (Zheng et al., 1999). However, the two plant v-SNAREs appear to function in different membrane transport processes. In this connection, AtVTI1a is capable of substituting for the yeast Vti1p, in Golgi-to-PVC transport, where as AtVTI1b can substitute in two alternative pathways: the vacuolar transport of alkaline phosphatase and the direct cytosol-to-vacuole pathway used by aminopeptidase I. In yeast, these multiple transport steps are mediated by a the same Vti1p (Fischer von Mollard and Stevens, 1999). Thus, the two related AtVTI1 proteins in Arabidopsis could be involved in vacuolar transport pathways that are unique in plants. An examination of the subcellular distribution of AtVTI1a indicated that it is localized at the TGN and the PVC containing the t-SNARE, AtPEP12p where AtVTI1a interacts with AtPEP12p *in*

vivo (Zheng et al., 1999). Finally, AtVTI1a colocalizes with the vacuolar sorting receptor, AtELP, at the TGN (Zheng et al., 1999). The colocalization of AtELP, AtVTI1a and AtPEP12p suggests that these three proteins are involved in the same pathway, a pathway that may be involved in the transport of a subset of proteins between the TGN and PVC in Arabidopsis. The function of either AtVTI1b or AtVAM3p is not known at this time.

A homolog of the yeast Vps45p (a Sec1p-related protein), AtVPS45p has been characterized in Arabidopsis (Bassham and Raikhel, 1998). Genetic and biochemical evidence shows that Vps45p interacts with both the t-SNAREs, Pep12p (at the PVC) and AtTLG2 (at the TGN) in yeast (Burd et al., 1997; Abeliovich et al., 1999). However, in subcellular fractionation studies of Arabidopsis roots, AtVAM3p was found to cofractionate only with AtELP, a marker for the TGN, but not with AtPEP12p present on the PVC. This result is further supported by some recent immunogold labeling studies where AtVPS45p has been found to colocalize with AtELP at the TGN in the same cells (Bassham, Kovalaeva and Raikhel, unpublished results). Further, AtVPS45p has been found to interact *in vitro* with the TGN-localized t-SNARE, AtTLG2a, but not with AtPEP12p from Arabidopsis (Bassham, Sanderfoot, Kovalaeva and Raikhel, unpublished results). These recent results indicate that the functional homology first indicated by the complementation of yeast mutants defective in components of the SNARE machinery may not indicate function of these homologs in the plant cell. Thus, although many of the basic components of the yeast SNARE machinery exists in plants, they may have evolved additional or different functions that are unique to plants. The present available evidence suggests that these identified plant vesicle transport components, together with AtELP,

play a role in targeting of proteins to the plant vacuole, via the NTPP-mediated pathway. However, more direct genetic or biochemical evidence is necessary, before any conclusion can be made regarding their biological function in vacuolar protein transport.

V. Conclusion

Our understanding of the components of the vacuolar transport machinery has come a long way since the identification and characterization of the first set of plant vacuolar targeting determinants. However, much of this knowledge comes from indirect evidence, that leaves the mechanism of protein transport to the plant vacuole still unclear. Although the basic machinery is likely to be conserved in plants, there already appears to be some components that are unique to plants. This is evident from the use of multiple targeting signals and transport pathways, for the delivery of proteins to functionally different vacuoles within the same plant cell. Identification of several homologous components of the yeast machinery has been very useful in understanding the basic machinery. However, future efforts must be directed towards isolating components that may be specific to plants, and in understanding their roles in vacuolar targeting.

VI. Thesis Scheme

The plant vacuole is an essential intracellular compartment that performs a wide range of biological functions. When I started this project, the goal was to isolate and characterize a potential vacuolar sorting receptor from Arabidopsis. At that time, several mammalian protein sorting receptors involved in endocytosis and lysosomal targeting, had already been identified and characterized at the molecular level. The gene encoding the yeast vacuolar sorting receptor, Vps10p, had just been cloned and characterized.

In plants, several different vacuolar sorting signals have been characterized in detail and vacuolar protein sorting has been shown to be saturable (Vitale and Raikhel, 1999). In addition, CCVs have been shown to be involved in protein transport to the plant vacuole. This indicate the involvement of a sorting receptor that may recognize specific sorting signals and interact with the signals during the sorting process mediated by CCVs. However, the molecular details of the transport machinery was not known. Although a putative vacuolar sorting receptor (BP-80) had been purified from pea CCVs, but it had not be characterized at the molecular level.

In an attempt to clone a potential sorting receptor from Arabidopsis, an extensive literature review was first carried out to study the characteristics of several different eukaryotic protein sorting receptors. Using a genomic approach, I was able to identify a family of cDNAs from the Arabidopsis database, that were related to several of the eukaryotic protein sorting receptors, specifically those involved in CCV-mediated protein transport (Chapter 2). One of these cDNAs, AtELP, was cloned and characterized in detail. The detailed subcellular distribution of the potential sorting receptor was determined by both biochemical fractionation and electron microscopy (Chapter 2 and 3). The next step of my project involved analysis of the function of AtELP in protein transport to the vacuole in Arabidopsis, using both *in vivo* and *in vitro* approaches (Chapter 4). The cloning, characterization and functional analysis of AtELP, has not only provided the plant cell biology community an important subcellular marker, but has contributed significantly to our understanding of the complex nature of the plant vacuolar sorting machinery.

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Chapter 2

Cloning and Subcellular Location of an *Arabidopsis* Receptor-Like Protein that Shares Common Features with Protein Sorting Receptors of Eukaryotic Cells

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ABSTRACT

Many receptors involved in clathrin-mediated protein transport through the endocytic and secretory pathways of yeast and animal cells share common features. They are all type I integral membrane proteins, containing cysteine-rich lumenal domains and cytoplasmic tails with tyrosine-containing sorting signals. The cysteine-rich domains are thought to be involved in ligand binding, whereas the cytoplasmic tyrosine motifs interact with clathrin-associated adaptor proteins during protein sorting along these pathways. In addition, tyrosine-containing signals are required for the retention and recycling of some of these membrane proteins to the *trans*-Golgi. Here, we report the characterization of an ~80-kD epidermal growth factor receptor (EGF)-like type I integral membrane protein containing all of these functional motifs from *Arabidopsis thaliana* (called AtELP for *Arabidopsis thaliana* EGF receptor-Like Protein). Biochemical analysis indicates that AtELP is a membrane protein found at high levels in the roots of both monocots and dicots. Subcellular fractionation studies indicate that the AtELP protein is present in two membrane fractions corresponding to a novel undefined compartment, and a fraction enriched in vesicles containing clathrin and its associated adaptor proteins. AtELP may therefore serve as a marker for compartments involved in intracellular protein trafficking in the plant cell.

INTRODUCTION

Proteins that are transported through the secretory pathway in eukaryotic cells are imported into the endoplasmic reticulum (ER) and travel through the Golgi apparatus. Upon arrival at the *trans*-Golgi network (TGN), vacuolar and secreted proteins are sorted and transported to their respective destinations (Bar-Peled et al., 1996; Rothman, 1996). The transport of many of these cargo proteins through the secretory pathway is mediated by small vesicles, a process requiring specific soluble and membrane proteins (Rothman and Wieland, 1996). During the transport process, vesicles carrying cargo first bud from the donor membrane, then travel to and dock at the target membrane, finally fusing to deliver the cargo (reviewed in Bassham and Raikhel, 1996; Rothman and Wieland, 1996).

Among the different types of vesicles associated with the transport of proteins along the secretory pathway in eukaryotic cells, clathrin-coated vesicles (CCVs) have been best characterized. CCVs play an important role in receptor-mediated endocytosis (reviewed in Pearse and Robinson, 1990; Schekman and Orci, 1996) and in targeting of proteins to the lysosome or vacuole (Dahms et al., 1989; Pryer et al., 1992; Hohl et al., 1996). During receptor-mediated endocytosis in animal cells, tyrosine-based sorting signals in the cytoplasmic tails of cell surface receptors associate with a cytosolic adaptor protein complex (AP2) at the plasma membrane (PM). Subsequent interaction of the receptor-adaptin complex with clathrin initiates the formation of CCVs (Pearse and Robinson, 1990). These vesicles then bud from the PM and fuse with the endosomal membrane, where the receptor is delivered for further transport to the lysosome for degradation or recycling back to the PM.

Using biochemical (Glickman et al., 1989; Dahms et al., 1989) and genetic (Seeger and Payne, 1992) means, transport of some proteins from the TGN to the vacuole or PM has been shown to require CCVs. At the TGN, a different set of cytosolic adaptor complex proteins (AP1) are thought to interact with cytoplasmic tyrosine motifs of

transmembrane proteins such as the mannose-6-phosphate receptor (M-6-PR) in forming CCVs for transport to the mammalian lysosome (Glickman et al., 1989). In yeast, homologs of the mammalian clathrin heavy chain (Lemmon & Jones, 1987) and several of the adaptin genes have been identified, and their involvement in vacuolar targeting has been demonstrated genetically (Rad et al., 1995; Stepp et al., 1995).

In plants, the involvement of CCVs during endocytosis at the PM of rapidly growing cells has been described in a number of studies (reviewed in Low & Chandra, 1994), and their involvement in membrane recycling from the PM has been suggested during cell-plate formation and growth of pollen tube tips (Samuels et al., 1995; Blackbourn and Jackson, 1996). In addition, CCVs are associated with the plant TGN (Staehelin et al., 1990; Driouich, 1993), and CCV-enriched fractions were found to contain precursors of some vacuolar seed storage proteins (Harley and Beevers, 1989; Hoh et al., 1991). However, a more recent study has reported that it is complex glycoproteins, not storage proteins, that are carried by CCVs (Hohl et al., 1996). Nevertheless, a number of studies have provided definitive evidence for the participation of CCVs in intracellular protein transport in plants. These include the recent cloning of the clathrin heavy chain gene from soybean and the localization of the protein to the PM (Blackbourn and Jackson, 1996), the identification of a β-type adaptin from zucchini (Holstein et al., 1994), the cloning of a σ-adaptin from *Camptotheca acuminata* (Maldonado-Mendoza and Nessler, 1996), and the isolation of a putative vacuolar targeting receptor (BP-80; Kirsch et al., 1994a) having a broad binding specificity for plant vacuolar targeting signals from pea CCVs (Kirsch et al., 1996).

Our laboratory is currently pursuing biochemical and genetic approaches to identify components of the protein sorting machinery of the plant secretory pathway; however, we have adopted an additional method for the isolation of gene (s) that may encode some components of the machinery. This approach is based on the use of known functional motifs present in many of the receptor proteins involved in clathrin-dependent

intracellular protein sorting in mammalian and yeast cells. We report the characterization of AtELP (*Arabidopsis thaliana* EGF receptor-Like Protein), an ~80-kD type I integral membrane protein whose predicted structural organization is very similar to that of mammalian and yeast protein-sorting receptors. AtELP is found in a coated vesicle population enriched for CCVs. Furthermore, it appears to be localized to a novel intermediate compartment that may correspond to the plant counterpart of the mammalian or yeast endosome.

MATERIALS AND METHODS

Plant Growth

Ten mL of *Arabidopsis thaliana* ecotype Columbia cell suspension line (T87-C33, a generous gift from Dr. Michael Axelos) were subcultured in 50 mL of media (0.32 g/L Gamborg's B-5 with minimal organic, 20 g/L sucrose, 1 mg/L 2.4-D at pH 5.7) and were grown on a rotary shaker at 50 rpm at 22°C under light conditions as described in Bar-Peled and Raikhel, 1997. Seeds of *Arabidopsis thaliana* ecotype Columbia (~15 seeds) were sterilized and placed in a 125-mL flask containing 50 mL GM liquid medium [4.3 g/L Murashige & Skoog salts mixture (Gibco-BRL), 0.5 g/L MES, 10 g sucrose, 0.1 g /L myo-inositol, 100 ml/L of 10 mg/mL thiamine-HCl, 50 ml/L of 10 mg/mL pyridoxine and 50 ml/L of 10 mg/mL nicotinic acid, adjusted to pH 5.7 with KOH]. The sterile plants were germinated and grown in the flasks placed on a rotary shaker (50 rpm) in a 22°C incubator under a 12-h fluorescent light/dark cycle. To study tissue-specific protein expression patterns, seeds were germinated either in GM-agar plates and grown as described above, or in pots and grown in a controlled environment chamber at 22°C under a 12-h day/dark cycle: 120 mmol m^{-2} s^{-1} light intensity, 70% relative humidity.

Plant materials (roots, leaves, flowers, pollen, stems, and siliques) were collected at different times as indicated, and analyzed.

Identification and Cloning of *AtELP*

A search of the plant EST databases using the Motif Explorer program (provided by the Arabidopsis cDNA Sequence Analysis Project at Michigan State University) identified the products of the AtELP gene from *A. thaliana*. The Motif Explorer tool was developed by the Data Acquisition, Analysis and Distribution Project at the University of Minnesota (Bieganski 1995). To achieve this, we performed computer searches with various consensus sequences (Tyr-motif and various Cys-rich motifs) of receptors involved in endoctyosis and protein sorting. Sequence alignment of the Cys-rich B.2 motif of the animal endocytic receptor, low-density lipoprotein receptor superfamily contains a consensus sequence with the longest uninterrupted stretch of five amino acids (NNGGCS). Many variations of this consensus sequence occur in various proteins and we searched the Arabidopsis EST data base at monthly intervals with a number of variations of this peptide sequence. Specifically, a search of the Arabidopsis EST data base with the "NNGGC" sequence, using the Motif Explorer program identified two ESTs (nos. T22799 and T43896). BLAST analysis of T22799 identified another Arabidopsis EST (no. R29853). A search of the GenBank at the National Center for Biotechnology Information (NCBI) with the sequence from R29853 identified two new Arabidopsis ESTs (nos. R90202 and T42090) and three rice ESTs (nos. D40971, D41266, and D40769). All of these ESTs were partial clones and were within the same region of a protein. We obtained all four Arabidopsis ESTs from the Michigan State University-Department of Energy Plant Research Laboratory (MSU-DOE PRL) Genome

Sequencing Project (Newman et al., 1994), and upon restriction digest analysis, decided to concentrate our efforts on nos. R90202 and R29853.

To obtain a full-length version of R90202, we used an antisense primer corresponding to the 5' end of the R90202 clone, the T7 primer, and the PRL2 cDNA library (Newman et al., 1994) as template. A PCR product was isolated that had a partial 3' end sequence identical to the 5' end of R90202. Furthermore, the partial 5' end sequence of this PCR product was used to blast the Arabidopsis data base. The 5' end sequence identified two Arabidopsis EST clones (nos. R30384 and Z38123). We obtained R30384 from MSU-DOE PRL Genome Sequencing Project and found that it contained the largest insert of all of the five Arabidopsis EST clones (nos. T22799, R29853, R90202, T42090, and R30384). The clones R30384 and R90202 were sent for complete sequencing. Analysis of the sequence indicated that the two contiguous cDNAs shared a 130-bp overlapping region, and when fused, indicated the presence of an open reading frame of 623 amino acids, the exact size of BP-80, as reported by Paris and Rogers (1996). Using site-directed mutagenesis in the overlapping region of the two contiguous EST clones R30384 and R90202, we constructed the full-length *AtELP*. An extensive RNA and genomic DNA analysis (by PCR and Southern analysis using probes and primers from various regions of the two cDNAs) all indicated that the two contiguous cDNAs are derived from the same gene.

The full-length 2314-bp *AtELP* cDNA (pSA23) was constructed in pGEX-5X-1 (Pharmacia) by an in-frame fusion of two contiguous (130 bp overlap) *A. thaliana* cDNAs (EST clone nos. R90202 and R30384). DNA sequencing was performed at the W. M. Keck Foundation at Yale University. The plasmid pSA6 was constructed to

produce an in-frame fusion between GST and the C-terminal half of the ORF of *AtELP* (aa 323 - 623). All *AtELP* DNA fragments were inserted into pGEX vectors (Pharmacia).

Generation and Purification of GST Fusion Protein and Preparation of Antibodies

The plasmid pSA6 was constructed to produce an in-frame fusion between GST and the C-terminal half of the ORF of AtELP (aa 323 - 623). *E. coli* (strain DH5α) cells containing pSA6 were grown in 100 mL LB media at 37°C to OD_{600}=0.8-1. Protein expression of the GST-fusion construct was induced by adding 0.2 mM IPTG and shifting the cell culture to 28 °C. The soluble GST-fusion protein was purified by affinity chromatography as described (Bar-Peled and Raikhel, 1996). The fusion protein (~100 mg) was emulsified with Titer-Max in a total of 1 mL and injected into rabbits. The rabbits were boosted three more times each with 50 mg of GST-fusion proteins emulsified with Titer-Max (CytRx Corp.). Other antibodies used in this study were: anti AtSEC12 sera (Bar-Peled and Raikhel, 1997), anti RD28, and anti γ-TIP sera (kindly provided by Maarten Chrispeels), anti clathrin heavy chain sera (kindly provided by Tony Jackson), and anti β-adaptin sera (kindly provided by Margaret Robinson).

Northern Analysis

RNA was isolated from various tissues (see text) of *A. thaliana* ecotype Columbia as described (Bar-Peled et al., 1995). Equal amounts of RNA were fractionated on a 6% formaldehyde/1% agarose gel, blotted, and probed with [a-^{32} P]dATP (NEN) labeled DNA fragments from various regions of the *AtELP* cDNA that were generated with the Klenow fragment of DNA polymerase (Boehringer Mannheim) and random hexanucleotide primers.

Protoplast Preparation and Homogenization

Protoplasts were isolated from 4-day-old *A. thaliana* cell suspension cultures essentially as described (Bar-Peled and Raikhel, 1997) with minor modifications. Five

mL of drained cells from two flasks of 4-day-old *A. thaliana* cell suspension cultures were collected on a 94-mm filter. The cells were incubated with 15 mL of freshly made protoplasting solution [15.4% (w/v) sucrose, 0.32% (w/v) Gamborg's B-5 minimal organic, 100 mg caylase 345 L, 15 mg pectolyase Y-23] at room temperature for about 3 h on a rotary shaker (60 rpm). The treated suspension was filtered through a 94-mm metal screen, and the volume of the filtrate containing protoplasts was adjusted to about 40 mL with 15.4% (w/v) sucrose, 0.32% (w/v) Gamborg's B-5 minimal organic solution. The filtrate was poured into babcock centrifuge bottles, and protoplasts were separated from broken cells after centrifugation (1,100 rpm in IEC HNSII clinical centrifuge swinging bucket rotor, 10 min, 25°C). The floated protoplast band was collected and transferred to a centrifugation tube containing 20 mL 0.4 M betaine, 3 mM Mes, 10 mM CaCl2, pH 5.7. Protoplasts were pelleted (10 min at 50*g* at room temperature), resuspended in 4 mL of cold lysis buffer containing 20 mM HEPES-KOH, pH 7.1, 13.5% (w/v) sucrose, 10 mM KOAc, 1 mM DTT and 0.5 mM PMSF. EDTA (1 mM) or $MgCl_2$ (3 mM) were added when indicated. Protoplasts were lysed gently by at least eight passages through a 25 5/8-gauge needle and lysis was confirmed by microscopy.

Total Protein Isolation

One mL of packed protoplasts was passed eight times via a syringe equipped with a 25-5/8-gauge needle. Five mL of drained cell suspension cultures or plant tissues (up to 1 g) were ground on ice in a mortar with a pestle in the presence of 100 mg acid-washed glass beads. Total protein was then extracted by further grinding with 4 mL of cold lysis buffer (see above), containing 1 mM DTT and 0.5 mM PMSF. Where indicated, 3 mM $MgCl_2$ were included in the lysis buffer and EDTA was omitted. Samples were placed at 4°C on a shaker for up to 15 min while other samples were extracted. Samples were further lysed by passing the homogenate 8 times through a 25-5/8-gauge needle, and filtered through three layers of miracloth (25 mm) to remove unbroken cells. The homogenate was termed total crude homogenate.

Differential and Sucrose Density Gradient Centrifugation

The total crude homogenate was further centrifuged for 10 min at 1,000*g* (4 °C), and the top 3.5-mL of supernatant (termed S1) was saved. Total microsomes were prepared by centrifuging this post-nuclear S1 fraction (60 min at 125,000*g*, r=max) generating a total membrane pellet (pellet at 125,000*g*=P125) and soluble protein fraction (supernatant at 125,000*g*=S125). For differential centrifugation experiments, the S1 fraction was further centrifuged at 3,000*g*, generating a supernatant (S3) and pellet (P3) fraction. The S3 fraction was centrifuged again at 8,000*g* for 20 min, generating a supernatant (S8) and pellet (P8) fraction. The S8 fraction was centrifuged again for 20 min at 14,000*g* to generate S14 and P14 fractions. The S14 fraction was then centrifuged for 30 min at 55,000*g* to generate the S55 and P55 fractions. The S55 fraction was finally centrifuged (1 h at 125,000*g*) to generate the S125 and P125 fractions. All pellets (P1, P3, P8, P14, P55, and P125) were rinsed briefly with 1 mL of lysis buffer and further resuspended in lysis buffer containing 150 mM NaCl, 1% Triton X-100, and 1% sarkosyl. After 15-30 min, the solubilized pellets (P1, P3, P8, P14, P55, and P125) were spun for 15 min at 125,000*g* (4 °C) to remove undissolved matter, and the supernatant was saved for analysis.

For separation of endomembrane organelles based on their densities, protoplasts were lysed in a buffer containing 1 mM EDTA (S1E) or 3 mM $MgCl_2$ (S1M). After centrifugation (1,000*g* for 10 min at 4 °C), the supernatants in each homogenization buffer (S1E or S1M) were collected and applied to either linear 16-55% (w/v) sucrose gradient systems, or to step sucrose gradients. The linear sucrose gradients were buffered in 10 mM HEPES-KOH (pH 6.9), 10 mM KOAc containing either 2 mM EDTA or 5 mM $MgCl_2$, and were prepared in 12-ml Beckman ultra clear thin tubes that fit the SW40Ti rotor. The step 16-55% sucrose gradients were made from stock solutions containing 55%, 40%, 33.5%, 26.5%, 16% (w/v) sucrose in the above buffer containing EDTA or

$MgCl_2$, and were prepared by sequential layering of the following stocks into 12-ml Beckman ultra clear thin tubes: 0.75 mL of 55% sucrose solution, three 0.97 mL aliquots of 40% sucrose, three 0.77 mL aliquots of 33.5% sucrose, two 1-mL aliquots of 26.5% sucrose, and two 0.75-mL aliquots of 16% sucrose solution. Three mL of either S1E or S1M were layered on top of the 9-mL linear- or step-sucrose gradient which was made in the presence of 1 mM EDTA or 3 mM $MgCl_2$, respectively. Gradients were centrifuged for 2 h at 150,000*g* in a Beckman SW40Ti rotor, at 4 °C. Fractions were collected from the top and the sucrose concentration was determined by measurement of the refractive index. Aliquots (60 mL) of the fractions were separated by SDS-PAGE and the distribution of AtELP and other marker proteins was analyzed by immunoblotting.

Golgi Marker Enzyme Assay

The assay for the activity of the Golgi marker enzyme, xyloglucan 1,2-α-L-fucosyl-transferase, was carried out essentially as described (Hanna et al., 1991). The standard assay system consisted of 200 mL of a buffer containing 25 mM PIPES-KOH, pH 6.5, 2 mM $MgCl_2$, 0.5 mg/mL purified soluble tamarind xyloglucan, 0.5% Triton X-114, 0.7 mM GDP-^{3}H-fucose (740 Bq/fraction), and 100 mL protein sample solubilized with Triton X-114 (final 1%) from fractions of the sucrose density gradients were prepared in the presence of EDTA. The mixed enzyme reactions were allowed to proceed at room temperature for 30-120 min and were then terminated by the addition of 0.5 mL of 95% (v/v) ethanol to a final concentration of 70%. After chilling for 1-2 h on ice, the reaction mixes were centrifuged at 14,000*g* at room temperature to pellet the radiolabeled products. The resulting insoluble pellet was washed twice with 1.2 mL of 70% (v/v) ethanol for 2-3 min to remove unreacted substrate and its breakdown products. The amount of [^{3}H] fucosylated xyloglucan product was assayed by liquid scintillation spectroscopy. The activity (in dpm) of the enzyme in each sucrose fraction was measured after subtraction of blanks lacking enzyme performed with each assay.

Isolation and Purification of Coated Vesicles from Developing Pea Cotyledons

Pea CCVs were isolated and purified as described (Harley and Beevers, 1989; Lin et al., 1992). Pea pods from the field or greenhouse-grown plants were harvested and the developing seeds (150 g) were collected and either used fresh or stored at -80 °C until use. The final coated vesicle (CV) preparation consisted of a green pellet (CVI) and a yellow-green region (CVII) above the pellet. CVs recovered from each of the regions were diluted and pelleted after further centrifugation at 120,000*g* for 1 hr in a Beckman 70Ti rotor. The suspended CVs of each fraction were aliquoted and frozen at -70 °C for further analysis by immunoblotting.

SDS-PAGE and Immunoblotting

Proteins were quantified according to the method of Bradford, 1976, using BSA as a standard. Protein molecular weight standards used as markers were purchased from BioRad (Broad-range marker, Catalog No. 72807A). Proteins (30-50 mg/lane) were separated on either 10 or 12% modified Laemlli-reduced SDS-PAGE (Bar-Peled et al., 1991), and transferred to a nitrocellulose membrane (0.45 mm), in 10 mM Tris, 100 mM glycine, 0.05% SDS, 10% methanol. Blots were stained with Ponceau S, incubated with blocking solutions [5% (w/v) milk powder in TBST (1X Tris-Buffer Saline/0.1% Tween-20)] for 2 to 12 h, reacted with primary antibody at 1:100 to 1:500 dilution, washed, and then reacted with secondary antibody (1:3000) conjugated to alkaline-phosphatase. Immune complexes were detected by color assay using NBT/BCIP as substrates.

RESULTS

Identification of an *Arabidopsis* cDNA Encoding an EGF Receptor-like Protein

In animal cells, cysteine-rich regions have been suggested to be involved in receptor-ligand interactions at the cell surface during CCV-mediated endocytosis (Yamamoto et al., 1984; Lalazar et al., 1988). The mammalian EGFR is a large integral membrane protein containing several cysteine-rich repeats (called EGF-repeats) in the extracellular

domain that are involved in interactions with its ligand (reviewed in Carpenter, 1987). A specific pattern of cysteine residues, termed class A or B cysteine-rich repeats, have been identified within these regions of the EGFR, LDLR, and related proteins (LRPs) (Stanley et al., 1986; Herz et al., 1988). Class B repeats fall into two subclasses (B.1 and B.2) based on the position of the cysteines within the repeats. Initially, using sequences from different regions of the well studied EGFR, LDLR and LRPs, we were unable to detect the presence of any related EST in Arabidopsis. We therefore decided to search the database with shorter sequences within the cysteine-rich repeats. A close analysis of the different class B cysteine-rich repeats of several of the eukaryotic receptors identified a highly conserved small pentapeptide sequence of "NNGGC" present in the class B.2 cysteine-rich EGF-repeats of the mammalian LDL receptors and related proteins (LRPs; Herz et al., 1988).

A search of the *Arabidopsis* EST database using the short "NNGGC" motif and the Motif Explorer program resulted in the eventual isolation of two contiguous cDNAs that were fused to construct a 2314-bp clone (*AtELP*) containing an ORF of 623 amino acids (aa) with a predicted molecular mass of ~69 kD (Figure 2.1A). The detailed process of identification and cloning of AtELP is presented in Methods and Materials. Southern blot analysis and PCR amplification of genomic DNA with primers specific to different regions of the two contiguous cDNAs, and northern blot analyses of RNA prepared from *A. thaliana* that were probed with various regions of the two cDNAs, all indicated the presence of a single band of the expected size corresponding to the cDNAs (data not shown). Hydropathy analysis (Kyte and Doolittle, 1982) of the predicted amino acid sequence (Figure 2.1B) indicated the presence of two hydrophobic regions (underlined in

Figure 2.1
Predicted sequence and structure of the AtELP gene product. (A) The predicted amino acid sequence of the 623-residue polypeptide encoded by *AtELP* (GenBank Acession No. U86700). The predicted N-terminal signal sequence, the EGF-repeats, transmembrane domain and the cytoplasmic tyrosine motif are underlined. (B) Hydropathy plot of the predicted amino acid sequence of AtELP using the method of Kyte and Doolittle (1982). (C) Predicted structural organization of the different domains of AtELP. "SS", signal sequence.

A.

```
MKLGLFTLSF LLILMLAMGR FVVEKNNLKV TSPDSIKGIY ECAIGNFGVP   50
   Signal Sequence

QYGGTLVGTV VYPKSNQKAC KSYSDFDISF KSKPGRLPTF VLIDRGDCYF  100

TLKAWIAQQA GAAAILVADS KAEPLITMDT PEEDKSDADY LQNITIPSAL  150

ITKTLGDSIK SALSGGDMVN MKLDWTESVP HPDERVEYEL WTNSNDECGK  200

KCDTQIEFLK NFKGAAQILE KGGHTQFTPH YITWYCPEAF TLSKQCKSQC  250

INHGRYCAPD PEQDFTKGYD GKDVVVQNLR QACVYRVMND TGKPWVWWDY  300

VTDFAIRCPM KEKKYTKECA DGIIKSLGID LKKVDKCIGD PEADVENPVL  350

KAEQESQIGK GSRGDVTILP TLVVNNRQYR GKLEKGAVLK AMCSGFQEST  400

EPAICLTEDL ETNECLENNG GCWQDKAANI TACRDTFRGR LCECPTVQGV  450
                          EGF-repeat 1
KFVGDGYTHC KASGALHCGI NNGGCWRESR GGFTYSACVD DHSKDCKCPL  500
                          EGF-repeat 2
GFKGDGVKNC EDVDECKEKT VCQCPECKCK NTWGSYECSC SNGLLYMREH  550
                          EGF-repeat 3
DTCIGSGKVG TTKLSWSFLW ILIIGVGVAG LSGYAVYKYR IRSYMDAEIR  600
                Transmembrane domain
GIMAQYMPLE SQPPNTSGHH MDI                               623
   Tyr-motif
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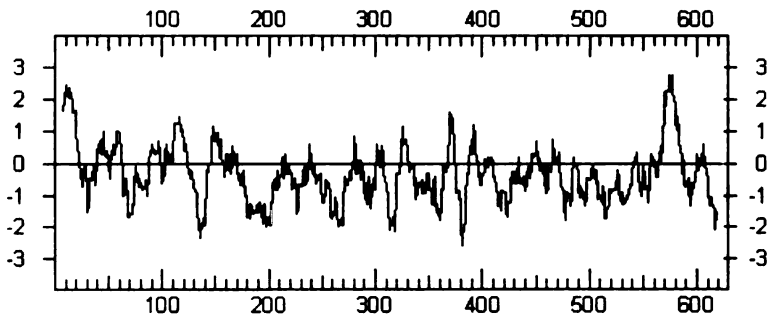
B.



C.

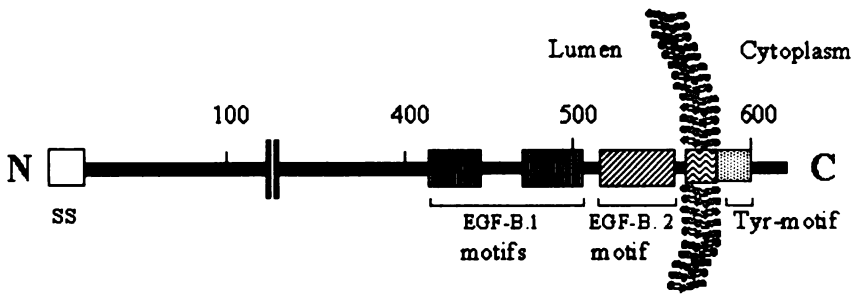


Figure 2.1A). The first region at the N-terminus, amino acids 1-19, encodes a potentially cleavable signal sequence for ER import as analyzed by the Signal P program (Nielsen et al., 1997; von Heijne, 1986). The second region starts ~60 residues upstream of the C-terminus (aa 564 - 587) and contains residues characteristic of membrane-spanning regions (Rao and Argos, 1986). Based on these observations, the cDNA was predicted to encode a type I integral membrane protein.

The rest of the AtELP protein is organized into domains (Figure 2.1C) that resemble those of the epidermal growth factor (EGF) and low density lipoprotein (LDL) receptors and its related proteins (Russell et al., 1984; Herz et al., 1988). A cysteine-rich (15%) region with identity to the class B cysteine-rich motifs of these mammalian receptors is found between aa 415-553 of AtELP. This presumed lumenal portion of the protein consists of three tandemly repeated EGF-like motifs (underlined in Figure 2.1A) that share identity (40%) with the class B (or EGF-like) motifs containing six cysteine residues in a span of 40 aa's (Herz et al., 1988). A potentially O-glycosylated region containing serine and threonine residues (aa 556 - 563) is present between the third EGF-repeat and the transmembrane domain (Figure 2.1A), This corresponding domain of the LDL receptor has many clustered O-linked carbohydrate chains (Russell et al., 1984). In addition, there are three N-glycosylation sites present in the putative lumenal portion of the protein.

The sequence of the predicted cytoplasmic domain of AtELP has similarity to the yeast Vps10 protein (Marcusson et al., 1994). Within this region of the protein are two tyrosine motifs (Figure 2.1A and C). The first tyrosine-containing region (aa 589 - 600) shares homology with a tyrosine-rich region that serves as a sorting signal in several

membrane receptors involved in protein trafficking, such as the yeast CPY vacuolar receptor (Vps 10p; Lin Cereghino et al., 1995; Cooper and Stevens, 1996) and the mammalian M-6-PR (Rohrer et al., 1995). The second tyrosine-containing region found between 606 and 609 resembles a Yxxϕ motif found on receptors that interacts with the PM or TGN adaptor protein complexes during the formation of clathrin-coated vesicles (Glickman et al., 1989; Ohno et al., 1995). The structural organization of these motifs in the deduced amino acid sequence encoded by the *AtELP* cDNA indicates that it encodes an EGFR-like protein that may be involved in clathrin-dependent intracellular protein trafficking in plants.

AtELP is Membrane Associated and Conserved between Monocots and Dicots

To characterize the protein encoded by the *AtELP* cDNA, the C-terminal half of the protein (aa 363 - 623) was expressed as a GST-fusion protein in *Escherichia coli*. The GST-fusion protein was purified by affinity chromatography over a glutathione Sepharose column and used to raise a rabbit antiserum. To investigate the tissue-specificity of the encoded protein, the serum was used to probe extracts from various *Arabidopsis* tissues (roots, leaves, stems, flowers, and siliques) and cell suspension cultures. Three proteins of ~80 kD were detected by the serum in each tissue examined on immunoblots (Figure 2.2A). The use of protease inhibitors was not able to reduce the relative intensity of any of the three proteins detected (data not shown), suggesting that they were unlikely to be degradation products of the same protein. Although the predicted molecular mass of the mature protein (aa 20 - 623) was ~67 kD, its apparent mass of 80 kD on immunoblots may be the result of post-translational modifications of the same protein. Alternatively, the three polypeptides could represent homologs of the

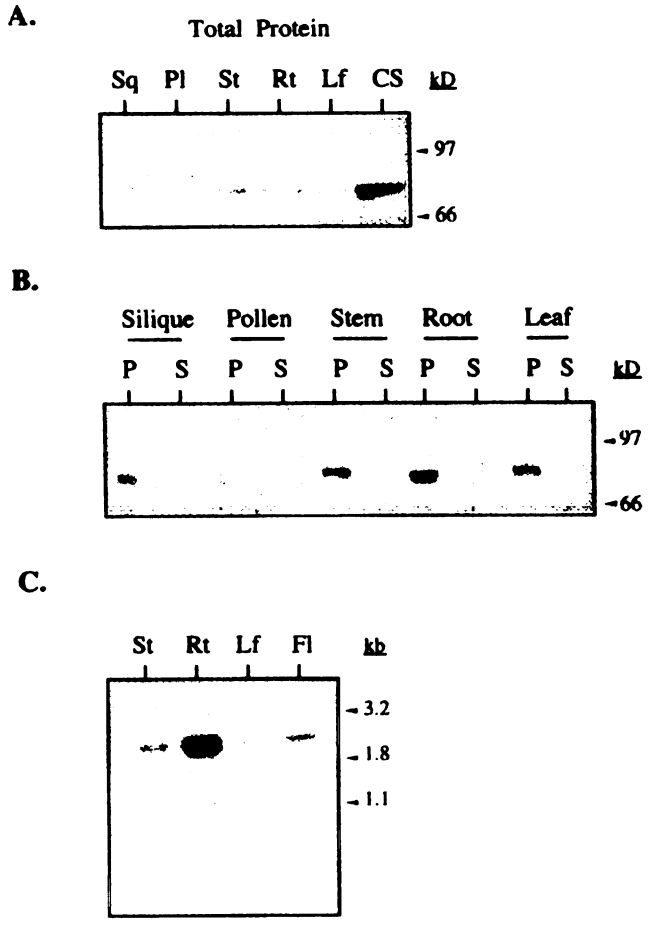


Figure 2.2
Distribution of AtELP in *A. thaliana* tissues. (A) Total protein extracted from green *A. thaliana* siliques (Sq), pollen (Pl), or stems (St) of mature plants, roots (Rt), or leaves (Lf) from two-week-old plants or *A. thaliana* cell suspension culture (CS). Equal amounts (50 μg) of proteins were separated on SDS-PAGE and immunoblotted with anti-AtELP antibodies. (B) Total protein extracted from the tissues in (A) was further fractionated by centrifugation at 125,000*g* to generate total membrane (P) and soluble (S) fractions. Western blots were probed with anti-AtELP antibodies. The size of AtELP is about 80 kD by comparison to molecular weight standards, indicated on the right. (C) Northern blot analysis of the AtELP transcript. Total RNA (30 μg) from stems (St), roots (Rt), leaves (Lf), and flowers (Fl) was separated in an agarose/ formaldehyde gel and blotted onto a nylon membrane. The membrane was hybridized with a ^{32}P- labeled fragment of the cDNA containing the AtELP open reading frame. Under high stringency conditions, using RNA from the tissues described, a single band of about 2.3 kb was found to hybridize with the probes made from different regions of the *AtELP* cDNA.
*Note: The AtELP antisera detected 3 polypeptides in the range of 80 kD. For a better resolution please refer to Figure 2.5 or 2.6.

AtELP protein. Consistent with the later possibility, during the *A. thaliana* database search that identified *AtELP*, we identified 3 additional partial EST clones sharing homology with *AtELP* between amino acids 300 and 460 (accession nos. T42090, T22799, and R29853).

Hydropathy plot analysis of the deduced amino acid sequence of *AtELP* (Figure 2.1B) predicted that it is a membrane protein. To confirm this prediction, the intracellular distribution of AtELP was examined by isolating microsomal fractions at 125,000 *g* and by immunoblot assays of proteins from total soluble (S), and total membrane (P) fractions from various *Arabidopsis* tissues (Figure 2.2B). The AtELP antibody detected proteins only in membrane-containing fractions, suggesting that AtELP is a membrane-associated protein. The amount of AtELP in microsomal fractions prepared from roots and rapidly dividing cell suspension cultures was higher than in leaves, stems, siliques or pollen, reflecting the distribution of its RNA as found by northern blot analysis (Figure 2.2C). This expression pattern (i.e. highest level in roots compared to leaves, stems or flowers) appears to be similar to that of a number of other proteins believed to be involved in the secretory pathway in plants, such as AtERD2, AtSAR1 (Bar-Peled et al., 1995; Bar-Peled and Raikhel, 1997) and AtPEP12 (Bassham et al., 1995; Conceição et al., 1997).

Using the *AtELP* sequence, homologs of AtELP were found in the rice (*Oryza sativa*) and maize (*Zea mays*) EST databases. The partial rice EST clone (No. D40971) shared homology with AtELP in the second EGF motif, whereas the partial maize EST clone (No. T18301) had homology in the EGF motif, the transmembrane domain, and the cytoplasmic tail containing the tyrosine motif (Figs. 2.1A and 2.3A). To examine the expression of AtELP homologs in monocots and dicots, microsomal fractions were

A.

```
AtELP       CSGFQESTEP AICLTEDLET NECLENNGGC WQDKAANITA CRDTFRGRLC 443
Rice  EST   -A--R-T---  -V--S--IQ- ---------- --------S- -K----
AtELP       ECPTVQGVKF VGDGYTHCKA SGALHCGINN GGCWRESRGG FTYSACVDDH 493
Maize EST                                             T-P- K-I---SNEI
AtELP       SKDCKCPLGF KGDGVKNCED VDECKEKTVC QCPECKCKNT WGSYECSCSN 543
Maize EST   -EG----V--  ----E-S---  I---Q--LY- --KG-S----  --------GD
AtELP       *GLLYMREHD TCIGSGKVGT TKLSWSFLWI LIIGVGVAGL SGYAVYKYRI 592
Maize EST   DNM-------  ---SKEGTA- -VG*-----V IFF-LVF--V GHTL------
AtELP       RSYMDAEIRG IMAQYMPLES QPPNTSGHHM DI                    623
Maize EST   -----S---A  --------DN -
```

B.

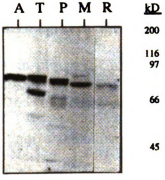


Figure 2.3
Homologs of AtELP are present in dicots and monocots. (A) Amino acid sequence comparison between AtELP and EST clones from the rice and maize databases. Horizontal lines represent residues identical to AtELP. Stars mark gaps introduced into the sequence to allow maximum homology. (B) Immunoblot analysis of AtELP homologs in dicots and monocots. Equal amounts of microsomal fractions prepared from roots of *A. thaliana* (A), tobacco (T), pea (P), maize (M), and rice (R) were analyzed by immunoblotting with anti-AtELP antibodies.

prepared from roots of *Arabidopsis*, tobacco, pea, maize, and rice (Figure 2.3B). In all the species tested, 3 cross-reactive proteins of ~80 kD were detected with the AtELP antisera similar to the results in *A. thaliana*. However, a weak cross-reacting band of the correct size (~80 kD) was detected in rice with the AtELP antisera, even though the same amount of protein was loaded on the gel. In addition, some other small cross-reacting proteins were detected by the serum in tobacco and pea. This indicates that AtELP may be conserved between monocots and dicots, suggesting an important role for the protein.

AtELP is an Integral Membrane Protein found in a Novel Compartment

To confirm our prediction that AtELP was an integral membrane protein, various conditions and treatments to extract the protein from the microsomal pellet were examined. Equivalent amounts of microsomal protein pellet (P125) were resuspended in lysis buffer alone (Figure 2.4), or in lysis buffer containing up to 2M urea, in 0.1 M Na_2CO_3, lysis buffer containing the ionic or non-ionic detergents Sarkosyl or Triton X-100 at 0.1 and 1% respectively, or in lysis buffer supplemented with NaCl at 1, 0.5 and 0.25 M (Figure 2.4), and in lysis buffer containing Sarkosyl and Triton X-100 and NaCl. After incubation on ice for 30 min, the mixtures were centrifuged at 125,000*g*. The content of AtELP in the resulting supernatants was determined by immunoblotting assays after SDS-PAGE. Treatments known to be effective in extracting proteins peripherally associated with membranes (urea, salt, and alkaline conditions) (Fujiki et al., 1982) were not effective in extracting AtELP from the membranes. Only non-ionic (Triton X-100) or ionic (sarkosyl) detergents extracted AtELP from the pelleted membranes (Figure 2.4), indicating that AtELP was an integral membrane protein.

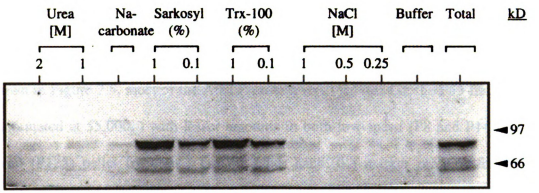


Figure 2.4

AtELP is an integral membrane protein. Microsomal fractions were prepared from a post nuclear (S1) fraction by centrifugation at 125,000*g* and resuspended in various buffers for 30 min on ice. The supernatants containing the resuspended pellets were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with anti-AtELP antibodies. Microsomes were resuspended in lysis buffer containing 1 or 2 M urea (Urea); only 0.1 M sodium carbonate (Na-carbonate), pH 11; lysis buffer containing: 1 and 0.1% sarkosyl; 1 and 0.1% Triton X-100 (Trx-100); 1, 0.5 and 0.25 M NaCl; lysis buffer alone (Buffer) or lysis buffer containing 150 mM NaCl, 1% sarkosyl and 1% Triton X-100 (Total). Protein samples were analyzed on a 12% SDS- PAGE gel instead of 10% as used for all other experiments. The additional bands towards the bottom of the gel are likely to be degradation products of AtELP.

To determine the association of AtELP with specific cell membranes, subcellular fractionation studies were first carried out using differential centrifugation, which allowed the separation of membrane-bound organelles based on their mass or density. Protoplasts were generated from actively dividing *A. thaliana* cell suspension cultures and gently lysed to minimize the disruption of the endomembrane system during the homogenization process. Lysates were fractionated by differential centrifugation into supernatant (S) and pellet (P) fractions. The relative amounts of AtELP, as well as marker proteins for various organelles were determined by immunoblot analysis. As shown in Figure 2.5, most of the AtELP protein was recovered in the P55 fraction (pellet fractionated at 55,000*g*) with lesser amounts in both low-speed (P8 and P14) and high-speed (P125) pellet fractions. On the other hand, the marker proteins for ER (BiP, Denecke et al., 1991; AtSEC12, Bar-Peled and Raikhel, 1997), tonoplast (γ-TIP, Gomez and Chrispeels, 1993), and PM (RD28, Daniels et al., 1994), were found mostly in the lower speed pellet fractions (P1, P3, P8, P14). Thus, AtELP had a different fractionation pattern from these endomembrane markers, suggesting that it was not localized in these organelles.

To gain additional information about the intracellular location of AtELP, subcellular fractionation studies were next carried out by equilibrium buoyant density centrifugation using the post-nuclear fraction (supernatant from a 1000*g* centrifugation) of protoplasts. This technique separates organelles on the basis of their inherent buoyant density differences and has been used to resolve the ER, mitochondria, PM, vacuole, and the Golgi complex in animal, yeast, and plant cells (Walworth and Novick, 1987; Beaumelle and Hopkins, 1989; Dewitt et al., 1996; Bar-Peled and Raikhel, 1997).

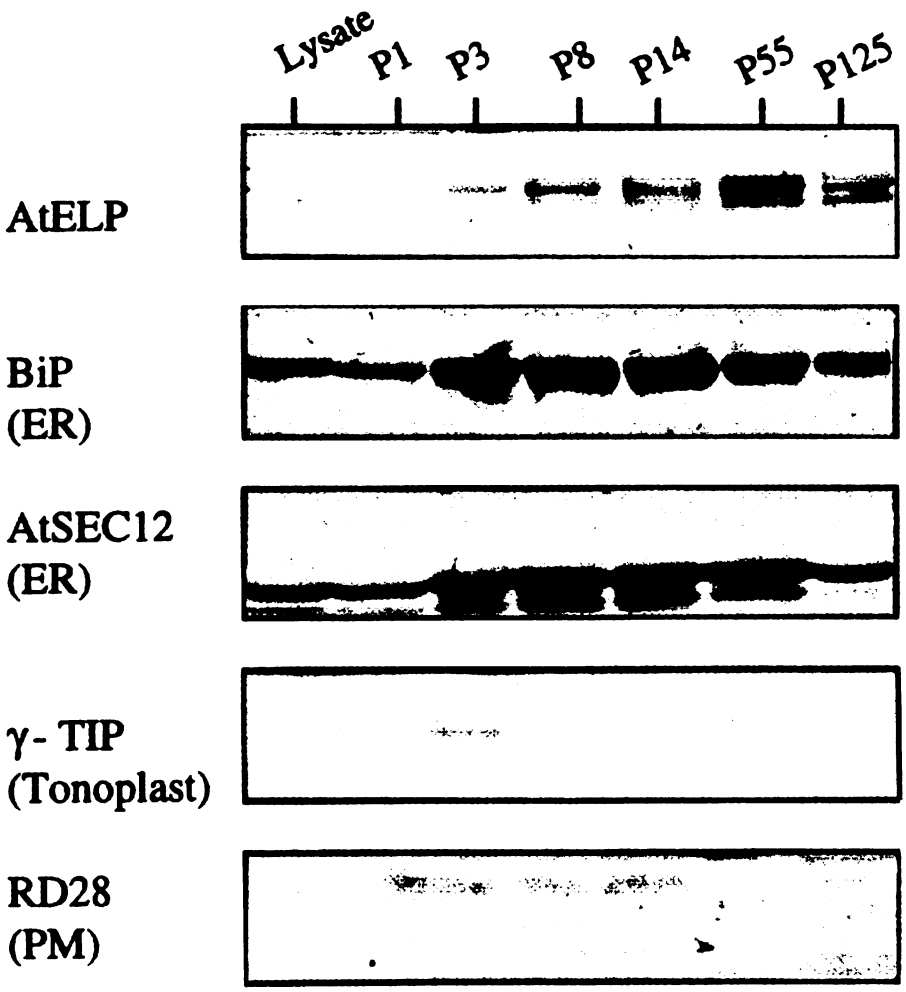


Figure 2.5
Subcellular fractionation of AtELP. Protoplasts isolated from *A. thaliana* cell suspension culture were lysed and fractionated by differential centrifugation as described in Materials and Methods. The lysate (total protein) was first subjected to a low-speed centrifugation at 1000*g*, yielding a pellet (P1) and a supernatant fraction that was subjected to further centrifugation at 3,000*g*, 8,000*g*, 14,000*g*, 55,000*g* and 125,000*g*, with the supernatant from each spin taken for use in the next centrifugation step. These differential centrifugation steps resulted in a P1, P3, P8, P14, P55, and P125 pellet, respectively. Equal amounts of protein (50 μg) from each of the pellet fractions were separated by SDS-PAGE, transferred to nitrocellulose, and cut into strips which were probed with antibodies against the organelle specific proteins BiP and AtSEC12 (ER), γ-TIP (tonoplast), and RD28 (PM).

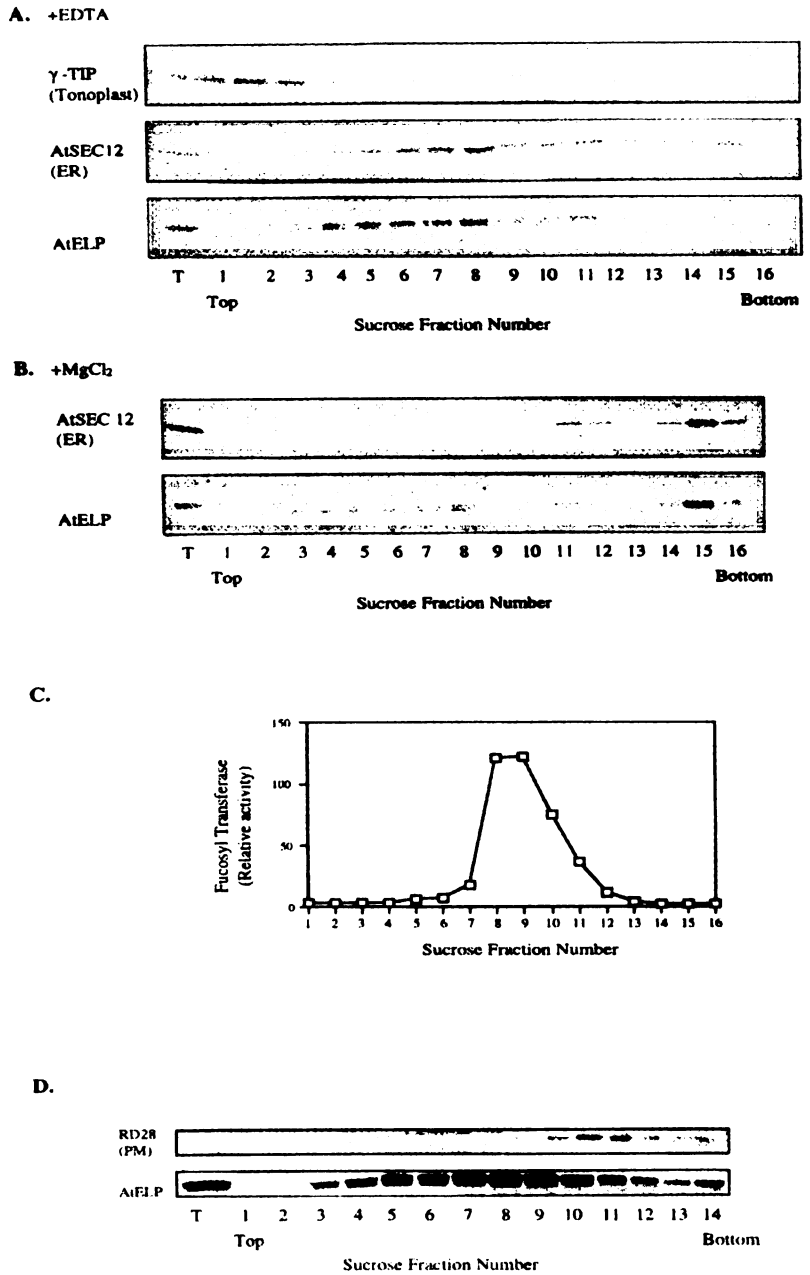


Figure 2.6

Sucrose density fractionation of AtELP. Protoplasts were made from *A. thaliana* suspension culture cells, resuspended in lysis buffer containing either 1 mM EDTA (A, C and D) or 3 mM $MgCl_2$ (B), and then lysed. The homogenates were centrifuged at 1,000*g* to remove nuclei, and the supernatant was loaded on a 16-55% step (A, B, and C) or a linear 16-55 % (D) sucrose gradient. After centrifugation, fractions were collected from the top to the bottom of the gradients, and 60 mL of aliquots from each fraction were separated on SDS-PAGE. Proteins were transferred to nitrocellulose membrane and immunoblotted (A, B and D) with antibodies against organelle specific proteins as in Figure 2.5. The activity of the Golgi marker enzyme xyloglucan fucosyltransferase (FT) in each fraction was measured separately and expressed as dpm/fraction and plotted (C).

Each fraction of the sucrose gradient was examined for the presence of AtELP and the endomembrane marker proteins g-TIP, AtSEC12 and RD28 by immunoblot analysis. Enzyme activity for xyloglucan 1,2-α-L-fucosyltransferase was also used as a marker for Golgi (Hanna et al., 1991). In step sucrose density gradients (Figure 2.6A, B and C), the vacuolar membrane (tonoplast)-specific marker γ-TIP fractionated at the top of the Suc gradients and peaked at approximately 16% Suc (fraction 3), as expected. The activity of the Golgi enzyme marker fucosyltransferase and the majority of the ER-marker, AtSEC12 both peaked at approximately 30% Suc, corresponding to fractions 8 and 9, respectively. The pattern of distribution of AtELP in the presence or absence of Mg^{2+} was different from the tonoplast, ER and Golgi markers. Significant amounts of AtELP were found in early fractions of the gradients (Nos. 4 and 5) corresponding to ~26.5% sucrose. In addition, AtELP was found in heavier fractions which contained the ER (Nos. 8, 11, and 15) and Golgi (No. 8) markers. Characteristic of ER-proteins (Lord, 1987; Bar-Peled and Raikhel, 1997), the AtSEC12 peak shifted completely from fraction 7 and 8 to a higher sucrose density corresponding to fractions 11 and 15, in the presence of Mg^{2+} (Figure 2.6B). However, in the presence of Mg^{2+}, the pattern of distribution of AtELP did not shift in the same manner as AtSEC12 (Figure 2.6A and B), although an increase in the amount of AtELP was observed in fraction 15. Much of AtELP appeared to remain in fractions 4-8 as found in the presence of EDTA. These results suggest that a major population of AtELP in *Arabidopsis* does not co-fractionate with the ER, Golgi or vacuolar membrane markers.

Because we were unable to resolve the PM from other endomembranes in the step sucrose gradients described in Figure 2.6A and B using the RD28 marker protein, a

separate linear sucrose density gradient was used to investigate the localization of AtELP to the PM. On linear sucrose density gradients (Figure 2.6D), the majority of the PM marker RD28 peaked at ~40% sucrose (fractions 10 and 11), whereas the peak for AtELP was found in a less dense fraction (Nos. 7, 8, and 9). Since AtELP had a different fractionation pattern than the available endomembrane markers for the tonoplast, ER, Golgi, or PM, we therefore propose that it is associated with a yet undefined intermediate compartment that is heavier than the vacuole and lighter than the ER, Golgi, or PM.

AtELP also Appears to be Enriched in Clathrin-coated Vesicles

The structural organization of the motifs present in the deduced amino acid sequence of the *AtELP* cDNA suggested that it encoded an EGF receptor-like protein that may be involved in clathrin-mediated intracellular protein sorting. While *A. thaliana* was found to be a poor source of CCVs (M. Bar-Peled, S. Ahmed and N. V. Raikhel, data not shown), developing pea cotyledons are a recognized source of these coated vesicles (Lin et al., 1992; Demmer et al., 1993). Furthermore, the AtELP antiserum was found to cross-react with a protein of ~80 kD in microsomal fractions prepared from pea roots (Figure 2.3B). To examine the association of AtELP with CCVs, we prepared coated vesicles that were enriched for CCVs from developing pea cotyledons. The protein profile of the purified coated vesicles on SDS-PAGE was very similar to that reported by others in plants (Lin et al., 1992; Demmer et al., 1993; Blackbourn and Jackson, 1996), with a dominant protein of 190 kD and others of 130 to 140 kD, 100 to 120 kD, and 50 to 55 kD. The 190-kD band corresponding to the clathrin heavy chain (see below) was particularly enriched in the coated vesicle II (CVII) fraction when compared to the CVI fraction or total protein (Figure 2.7A). Immunoblotting with antibodies to the soybean

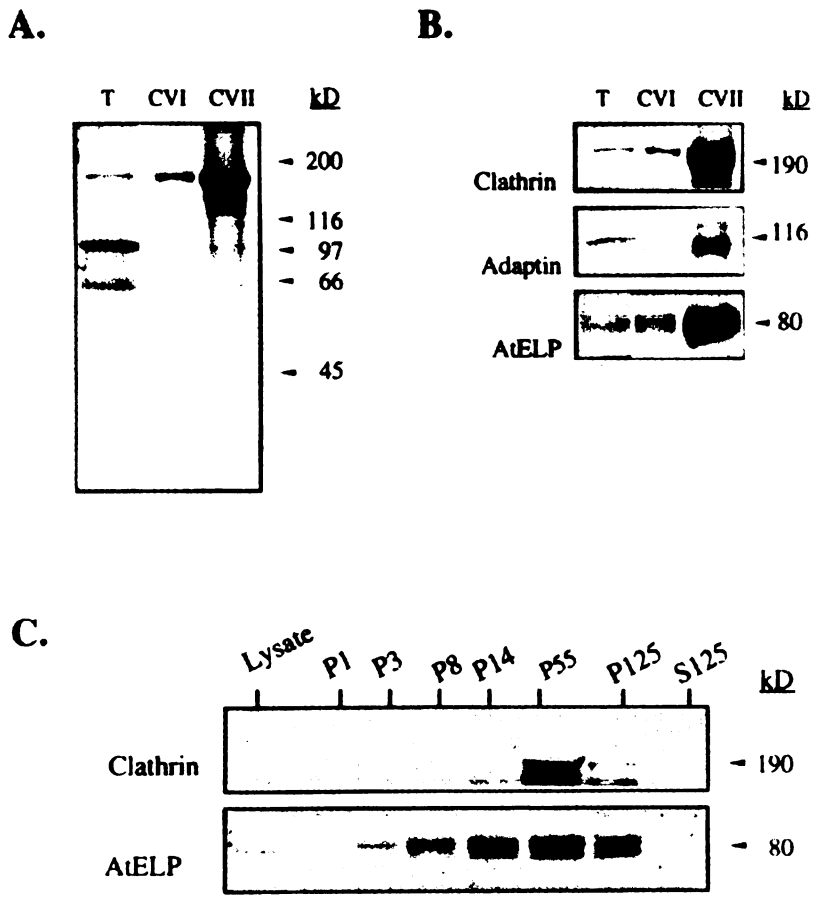


Figure 2.7
Enrichment of AtELP in clathrin-coated vesicles (CCVs). Coated vesicle preparations from developing pea cotyledons enriched for CCVs were analyzed by SDS-PAGE. (A) Coomassie Brilliant Blue stain of equal volumes of total homogenate (T) and coated-vesicle fractions CVI and CVII following electrophoresis. The molecular weight marker is on the right. (B) Western blot from (A) was probed with anti-AtELP, clathrin, and b-adaptin antibodies. The size of the organelle marker proteins were determined by comparison to molecular weight standards. (C) Pellet fractions from differential centrifugation experiments in *A. thaliana* as described in Figure 2.5 were separated on SDS-PAGE and analyzed by immunoblotting with anti-AtELP and anti-CHC antibodies. Each lane (lysate, P1, P3, P8, P14, P55, and P125) contains samples identical to those in Figure 2.5, in addition to a supernatant from the 125,000*g* centrifugation step (S125).

clathrin heavy chain (CHC; Blackbourn and Jackson, 1996) confirmed that the 190-kD band in the pea coated vesicle preparation was the clathrin heavy chain (Figure 2.7B). In addition, a 116-kD band corresponding to the plant clathrin-associated β-adaptin type protein was also enriched in these coated vesicles (CVII), and detected by antibodies to the mammalian b-adaptin (Robinson, 1987). In a similar manner, the AtELP homolog in pea was enriched in the coated vesicle preparations that were rich in clathrin and its associated adaptor protein. Surprisingly, the CVI fraction did not contain β-adaptin, although it contained clathrin and AtELP. However, other plant homologs of the mammalian clathrin-associated adaptor proteins of the AP1 or AP2 complex may be present in this fraction that could not be detected with the β-adaptin antisera in this fraction. Differential centrifugation experiments used to determine the association of AtELP with specific cellular membranes (Figure 2.5) in *A. thaliana* cell suspension cultures, indicated that the protein was associated with both low-speed (8,000*g* and 14,000*g*) and high-speed (55,000*g* and 125,000*g*) pellet fractions. To determine the association of clathrin with any of these fractions, subcellular fractions described in Figure 2.5 were probed with anti-AtELP and anti-CHC antibodies (Figure 2.7C). Although clathrin was found mostly in the high-speed (55,000*g* and some in the 125,000*g*) pellet fractions together with AtELP, it was not detectable in the low-speed (8,000*g* or 14,000*g*) pellet fractions, where significant amounts of AtELP were also present. These results, taken together with the membrane-association and subcellular fractionation data, suggest that AtELP may be present in the membranes of clathrin-coated vesicles, in addition to the intermediate compartment described above.

DISCUSSION

In this paper we have used a new method for the isolation of gene(s) that may be involved in the plant secretory pathway by searching the EST databases with known functional motifs present in receptor proteins involved in intracellular protein trafficking in other systems. A computer search carried out with the entire protein sequences of

many of these receptors (e.g. M-6-PR, Vps10p, LDLR, EGFR) was unable to identify any homology in the plant databases. However, the use of a short, highly conserved sequence of NNGGC identified a group of cDNAs from both the *A. thaliana* and *O. sativa* databases. Here, we have described a unique ~80-kD EGF receptor-like type I integral membrane protein from *A. thaliana*, AtELP. The amino acid sequence derived from the *AtELP* cDNAs shows the presence of several sequence motifs found primarily in mammalian and yeast integral membrane receptor proteins that are involved in intracellular protein sorting along the secretory and endocytic pathways. The AtELP protein appears to have four functional regions: i) a cleavable N-terminal signal peptide; ii) a lumenal region containing three cysteine-rich domains that shows homology to the EGF repeats; iii) a transmembrane segment; and iv) a cytoplasmic tail that contains two potential tyrosine motifs that could be required for clustering into CCVs or for Golgi retention/retrieval.

Cysteine-rich domains and/or EGF-like repeats have been found in the yeast vacuolar sorting receptor Vps10p (Marcusson et al., 1994), the human EGF and LDL receptors and their related proteins (Herz et al., 1988), and the thrombomodulin receptor (Wen et al., 1987). The cysteine-rich domains in the Vps10 protein may be involved in ligand binding (Horazdovsky et al., 1995); whereas in the LDL receptor they are believed to bind its ligands and apolipoproteins B and E (Yamamoto et al., 1984). In addition to the well-characterized EGF-EGF receptor interaction, in at least three cases (thrombomodulin, urokinase and Notch) the EGF repeats participate directly in protein-protein interactions (Kurosawa et al., 1988; Apella et al., 1987, 1988; Rebay et al., 1991). The only transmembrane protein with EGF repeats described in plants, Wak1, was identified recently in *A. thaliana* as a cell-wall-associated receptor-like protein kinase (He et al., 1996). The extracellular domain of the Wak1 protein contains several EGF repeats and may serve as a physical connection between the extracellular matrix and the

cytoplasm (He et al., 1996). It is therefore possible that the EGF-like regions in AtELP may also be responsible for interaction with a ligand.

The two tyrosine-containing regions in the putative cytoplasmic portion of AtELP may function as signals for targeting the protein. Tyrosine-based signals are important for the targeting, stability and function of a number of receptor proteins in both the mammalian and yeast secretory pathways (Bos et al., 1993; Rohrer et al., 1995; Lin Cereghino et al., 1995; Cooper and Stevens, 1996). The cytoplasmic tails of these membrane receptors contain tyrosine-based sorting signals (Yxxϕ, where Y represents a tyrosine, x any amino acid, and ϕ a hydrophobic amino acid) that allow the recruitment of the adaptor proteins to form the coated pits (reviewed in Sandoval and Bakke, 1994).

During receptor-mediated endocytosis in animal cells, cytoplasmic tyrosine-based sorting signals of cell surface receptors associate with a cytosolic adaptor protein complex (AP2-consisting of α-adaptin, β2-adaptin, μ2, and σ2) at the plasma membrane (PM). Subsequent interaction of the receptor-adaptin complex with clathrin initiates the formation of CCVs (Pearse and Robinson, 1990). These vesicles then bud from the PM and fuse with the endosomal membrane, where the receptor is delivered for further transport to the lysosome for degradation or recycling back to the PM.

In addition, some tyrosine motifs have been found to be important for the retention of membrane proteins at the TGN in mammalian and yeast cells (Bos et al., 1993; Voorhees et al., 1995; Wilcox et al., 1991) and most recently, a tyrosine-based targeting signal has been shown to mediate the sorting of an integral membrane glycoprotein into Golgi-derived CCVs (Höning et al., 1996). These cytoplasmic tyrosine motifs interact with other components of the sorting machinery, such as clathrin-associated AP1 adaptor protein complex (β1-adaptin, γ -adaptin, μ1, and σ1) at the TGN. The requirement for multiple tyrosine signals and their interaction with adaptor proteins at the late-Golgi has been shown in both animal and yeast cells during lysosomal or vacuolar targeting (Glickman et al., 1989; Lin Cereghino et al., 1995; Rad et al., 1995; Höning et al., 1996).

The first tyrosine-containing region (aa 589-594) in the cytoplasmic region of AtELP is adjacent to the transmembrane region and shows similarity to the tyrosine-based signals required for the targeting and function of the M-6-PR and Vps10p (Glickman et al., 1989; Lin Cereghino et al., 1995). The second cytoplasmic tyrosine motif, YMPL (aa 606 - 609), fits the consensus motif, YXXϕ. The YMPL motif resembles other tyrosine-based motifs implicated in targeting to endosomes or the TGN and interaction with the clathrin-associated adaptor proteins (Bos et al., 1993; Humphrey et al., 1993; Wong et al., 1993; Ohno et al., 1995). Both of the tyrosine-containing regions are conserved between AtELP and the maize EST clone.

Protein transport from both the PM and TGN to the vacuole/lysosome in yeast and animals proceeds via an intermediate endosome-like compartment (Vida et al., 1993; Gruenberg et al., 1989). The presence of multiple intermediate compartments (early, medial, or late-endosomes) between the Golgi, PM, and vacuole/lysosome is well documented in both mammalian and yeast cells (reviewed in Pryer et al., 1992; Vida et al., 1993). The transport processes between the Golgi or PM to the endosome are thought to be mediated by CCVs. The endosome to vacuole lysosome transport may also be vesicle-mediated. Alternatively, the endosome may fuse with the vacuole upon maturation. In plants, the presence of such intermediate compartments between the ER, Golgi, PM, and vacuole have been suggested in recent years. These include the rough ER (RER; reviewed in Okita and Rogers, 1996), partially coated reticulum (PCR; Hillmer et al., 1988; Griffing, 1991), and pre-vacuole (Conceição et al., 1997). Recently, some plant cells have been shown to contain two functionally different vacuoles at specific developmental stages that may later fuse to become a large vacuole (Hoh et al., 1995; Paris et al., 1996).

The results obtained from biochemical and subcellular fractionation studies of *A. thaliana* indicated that AtELP is an integral membrane protein that resides in at least two different membrane populations. One may correspond to a novel intermediate

compartment (fractionating at 26.5% sucrose) between the ER, Golgi, PM, and vacuole that could be a plant counterpart of the mammalian or yeast endosome. Interestingly, the density at which this compartment fractionates (26.5% sucrose) in sucrose gradients is similar to that reported for endosomes in cells of animal (Beevers, 1996) and yeast (Vida et al., 1993; Becherer et al., 1996), suggesting for the localization of AtELP in an endosome-like intermediate compartment in *A. thaliana*. Although some intermediate or post-Golgi compartments (some times also referred to as intermediate compartments) have been identified morphologically (Staehlin et al., 1990) or their presence suggested in some recent reviews (Okita and Rogers, 1996) no molecular markers for any of these compartments have been described yet, making it difficult for us to determine whether AtELP is present in any of those compartments. Thus, we refer to the AtELP compartment as a yet undefined novel intermediate compartment in the plant cell.

The other type of membrane containing AtELP may represent small transport vesicles such as clathrin-coated vesicles (CCVs). Indeed, AtELP co-fractionated with high-speed pellet fractions containing clathrin in subcellular fractionation studies, and the AtELP homolog in pea was enriched in CCVs prepared from developing cotyledons. Furthermore, these vesicle preparations were enriched for the clathrin-associated b-adaptin type protein. These results suggest that AtELP may also be associated with CCVs. This would be supported by the presence of a Yxxϕ motif in the putative cytoplasmic domain of AtELP, which may associate with clathrin via their interaction with adaptins during the formation of CCVs. The lack of marker proteins in pea prevented us from carrying out subcellular fractionation studies similar to those performed with *A. thaliana.*

The antibody raised against AtELP recognized three polypeptides in the range of ~80 kD. Although we can not determine at this point whether these polypeptides are all AtELP-like proteins or post-translational modifications of one (AtELP) protein, the three polypeptides always appeared to co-fractionate in all our subcellular fractionation studies.

Recently, L. Beevers (1996) and J. C. Rogers (Paris and Rogers, 1996) have indicated the presence of several 80-kD integral membrane proteins in pea and *Arabidopsis* with the domain structure and a subcellular location similar to that of AtELP. A member of this group of proteins isolated from pea CCVs (BP-80; Kirsch et al., 1994) has been shown to bind a broad range of plant vacuolar targeting signals (Kirsch et al., 1996). Similar to AtELP, the 80-kD protein from pea was not found to localize with ER or Golgi markers, but fractionated as a less dense compartment on sucrose density centrifugation experiments (Beevers, 1996; Okita and Rogers, 1996). However, when this less dense membrane fraction from pea cotyledons was incubated and allowed to associate with clathrin components, it sedimented in sucrose density gradients at a density equivalent to that of CCVs (Kirsch et al., 1994b).

AtELP is the first marker protein described that may reside in both an endosome-like intermediate compartment and CCVs in plants. The presence of AtELP in at least two different membrane populations may indicate that the protein is recycled between the membrane types. Interestingly, both the yeast Vps10p and the mammalian M-6-PR are type I integral membrane proteins required for the transport of multiple vacuolar/lysosomal hydrolases (Marcusson et al., 1994; Cooper and Stevens, 1996; Brown et al., 1986), and they cycle between the late-Golgi and endosomal compartments in their function as the sorting receptors for soluble vacuolar proteins. In addition, many of the membrane proteins (EGFR, LDLR, TfR) involved in receptor-mediated endocytosis are cycled between the PM and the endosome (reviewed in Pearse and Robinson, 1990).

In conclusion, the organization of the predicted functional domains of AtELP, its localization to an intermediate compartment, and association with CCVs are similar to several transmembrane receptor proteins that function at the PM or TGN during protein sorting along the mammalian and yeast endocytic or secretory pathways. We therefore propose that AtELP may play a similar role in intracellular protein trafficking in the plant

cell. Studies are currently underway to investigate this possibility, especially the role of AtELP in the trafficking of soluble vacuolar proteins containing the plant vacuolar targeting signals identified thus far (Chrispeels and Raikhel, 1992).

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Chapter 3

A Putative Vacuolar Cargo Receptor Partially Colocalizes with AtPEP12p on a Prevacuolar Compartment in *Arabidopsis* Roots.

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ABSTRACT

Targeting of protein cargo to the vacuole/lysosome is a multi-step process that appears to have conserved features between mammalian, yeast, and plant cells. In each case, some soluble vacuolar/lysosomal proteins are believed to be bound by transmembrane cargo receptors in the *trans*-Golgi network (TGN) that redirect these proteins into clathrin coated vesicles. These vesicles then appear to be transported to the prevacuole/endosome by a trafficking machinery that requires components identified in other vesicle targeting steps such as *N*-ethylmaleimide sensitive factor (NSF), soluble NSF attachment protein (SNAP), SNAP receptors (SNAREs), rab-type GTPases, and Sec1p homologs. Two likely members of this trafficking machinery have been characterized from *Arabidopsis thaliana*: AtPEP12p, a t-SNARE that resides on a what we now call a prevacuolar compartment; and AtELP, a protein that shares many common features with mammalian and yeast transmembrane cargo receptors. Here, we have further investigated the intracellular distribution of AtELP. We have found that AtELP is located at the *trans*-Golgi of *Arabidopsis* root cells, and that its C-terminus can preferentially interact *in vitro* with the mammalian TGN-specific AP-1 clathrin-adaptor complex, suggesting a likely role in clathrin coated vesicle-directed trafficking at the TGN. Further, consistent with a role in trafficking of vacuolar cargo, we have found that AtELP partially colocalizes with AtPEP12p on a prevacuolar compartment.

INTRODUCTION

Most vacuolar/lysosomal proteins first enter the secretory pathway at the endoplasmic reticulum (ER), then travel through the Golgi apparatus to the *trans*-Golgi network (TGN). At the TGN, vacuolar proteins are sorted away from secreted proteins due to the presence of positive vacuolar sorting signals (VSS). These VSS are posttranslationally-added mannose-6-phosphate (M-6-P) residues in mammalian cells, or specific protein sequences in plant and yeast cells (reviewed in Bassham and Raikhel, 1997, Robinson and Hinz, 1997). Plants appear to have a much more complex set of independent VSS, and the plant VSS do not function in yeast cells (reviewed in Bassham and Raikhel, 1997). This specificity may be due to the requirement for specific cargo receptors that recognize each VSS. These cargo receptors have been characterized from several eukaryotic cells, and include the mammalian M-6-P receptor (Kornfeld, 1992), the yeast carboxypeptidase Y (CPY) receptor (Vps10p; Marcusson et al., 1994), and the putative plant vacuolar sorting receptor (BP-80; Kirsch et al, 1994). Though overall sequence homology between these receptors is low, all are type-I membrane proteins which share common structural features such as lumenal cysteine-rich repeats, a single transmembrane spanning domain near the C-terminus, and the presence of Tyr-based motifs (Yxxϕ, where Y stands for Tyr, x for any residue, ϕ for a bulky-hydrophobic residue) on the cytoplasmic tail. In mammalian cells, soluble cargo bound for the lysosome is packaged into clathrin coated vesicles (CCVs; Traub and Kornfeld, 1997), and this is believed to be the case for soluble vacuolar hydrolases in yeast, as well (Seeger and Payne, 1992). In plant cells, it has been reported that some vacuolar-targeted proteins leave the TGN in CCVs (Harley and Beevers, 1989; Hohl et al., 1996); however,

trafficking of proteins to the plant vacuole is a complex process, and other vacuolar proteins have been shown to depart the TGN in vesicles that are different from CCVs (Hohl et al., 1996). Proteins destined for secretion have not been found to leave the TGN in CCVs (Robinson and Hinz, 1997; Traub and Kornfeld, 1997).

CCVs are formed at both the plasma membrane (PM) and the TGN. Research in mammalian cells has indicated that these can be differentiated due to specific clathrin adaptor complexes (APs) found in each. APs connect the clathrin coat to the vesicle membrane through interaction with the Tyr-motifs (or other signals) present in the cytoplasmic tail of receptors and other membrane proteins. Endocytosed proteins and receptors are packaged at the PM by AP-2, while proteins at the TGN are packaged by AP-1 (reviewed in Kirchhausen et al., 1997). Homologs of the mammalian AP complexes have been characterized in yeast (Phan et al., 1994; Rad et al., 1995; Stepp et al., 1995), and potential homologs of some proteins in the AP-complex have been identified in plants (Holstein et al., 1994; Maldonado-Mendoza and Nessler, 1996; 1997), suggesting that the adaptor specificity seen in mammalian cells may be conserved. Following budding, the coat disassembles and vesicles are apparently trafficked to the prevacuolar compartment (PVC) using machinery similar to that used in other vesicular trafficking steps such as *N*-ethylmaleimide sensitive factor (NSF), soluble NSF attachment protein (SNAP), SNAP receptors (SNAREs), rab-type GTPases, and Sec1p homologs (the SNARE hypothesis, reviewed in Bennett, 1995; Hay et al., 1997). This has been best shown in yeast for the transport of CPY by Vps10p: Vesicles that carry CPY are thought to contain the vesicle-(v-)SNARE Vti1p (von Mollard et al., 1997) which directs delivery to the PVC through interaction with the PVC target-(t-)SNARE Pep12p as well as several

soluble factors including NSF and the Sec1p-homolog Vps45p (von Mollard et al., 1997; Burd et al., 1997). CPY then continues on to the vacuole through a process which requires the vacuolar t-SNARE Vam3p (Darsow et al., 1997), while Vps10p (Cereghino et al., 1995; Cooper and Stevens, 1996) and presumably Vti1p (von Mollard et al., 1997) are recycled back to the Golgi apparatus.

Although the plant vacuolar targeting pathway appears to be much more complex than that found in yeast cells, both in the diversity of vacuolar sorting signals, as well as the type and number of vacuole-bound vesicles that leave the TGN, some conserved features will be found in all eukaryotic cells. Consistent with this hypothesis, several homologs of the machinery described above for yeast cells have been found in plants. AtPEP12p, an *Arabidopsis thaliana* homolog of yeast Pep12p, has been cloned and found to reside on a PVC in root cells (Bassham et al., 1995; Conceição et al., 1997). AtVAM3p, an *Arabidopsis* protein that complements a deletion mutant of the yeast vacuolar t-SNARE Vam3p has been identified (Sato et al., 1997). AtVPS45p, a Sec1p homolog from *Arabidopsis* that can functionally replace Vps45p in yeast has been recently characterized (Bassham and Raikhel, 1997). Further, a putative cargo receptor isolated from pea (*Pisium sativum*) CCVs that has been shown to bind the vacuolar sorting signals of some proteins (BP-80; Kirsch et al., 1994) has also been described. We have previously isolated AtELP, a protein from *Arabidopsis* which shares all the features common to cargo receptors found throughout eukaryotes (cysteine-rich repeats, single transmembrane domain, and cytoplasmic Tyr-based motifs) and is associated with CCVs isolated from peas (Ahmed et al., 1997). AtELP is highly homologous to BP-80, suggesting that it may also play a role in targeting of proteins to the plant vacuole, as has

been suggested for BP-80 (Kirsch et al., 1994; Paris et al., 1997). Here, we have further investigated the association of AtELP with CCVs, and have found that its cytoplasmic tail is capable of *in vitro* interaction with the proteins of the mammalian TGN-specific AP-1 adaptor complex. Further, through both biochemical approaches and immunoelectron microscopy, we have found that AtELP is localized on the *trans*-Golgi, as well as being colocalized with the t-SNARE AtPEP12p on the PVC of *Arabidopsis* root cells.

MATERIALS AND METHODS

In vitro AP-1 adaptor complex binding assay

AP-1 and AP-2 adaptor complexes were purified from calf brain coated vesicles as described previously (Rapoport et al., 1997; Rapoport et al., 1998). Details of the synthesis of the biotinylated photoactivatable cross-linking *Lamp1-YQTI and *TGN38-YQRL peptides are described elsewhere (Rapoport et al., 1997; Rapoport et al., 1998). Peptides corresponding to the cytoplasmic tail of AtELP (YMPL) and the Tyr-606 →Ala mutated form (AMPL) represent residues 599-612 within the AtELP sequence (Accession no. U86700) and were synthesized at the Macromolecular Structure Facility at Michigan State University using an Applied Biosystems 432A peptide synthesizer. The peptides were purified by gel filtration on a Biogel P2 column and analyzed by mass spectrometry and micro-sequencing. UV-induced cross-linking of the photoreactive peptides to purified AP complexes in the absence or presence of varying amounts of the AtELP competitor peptides was carried out as described (Rapoport et al., 1997). Following the cross-linking reaction, the cross-linked products were treated with sample buffer under reducing and denaturing conditions, analyzed by SDS-PAGE, and

transferred to nitrocellulose. The membranes were probed with streptavidin-horse radish peroxidase and detected by enhanced chemiluminescence.

Sucrose density gradients

Arabidopsis thaliana plants, ecotype RLD, were grown in liquid culture as described in ref. 25. Roots (~2 g) were separated from leaves and stems, chilled to 4°C, and ground in 6 ml of 50 mM Hepes-KOH, pH 6.5, 5 mM EDTA, 13.7% (w/v) sucrose, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol. The extracts were cleared at 2000*g*, prior to layering of 3 ml on top of a pre-formed step gradient consisting of the following steps: 1.0 ml 54%, 2.7 ml 40%, 2.2 ml 33%, 2.0 ml 24%, and 1.5 ml 15% (w/v) sucrose in 50 mM Hepes-KOH, pH 6.5, 5 mM EDTA. The gradients were spun in a Beckman SW40 swinging bucket rotor at 150 000*g* for 3 hr at 4°C. Twenty-four 0.5 ml fractions were collected from the top and sucrose concentrations for each fraction were determined with a refractometer. All fractions were then precipitated with 10% trichloroacetic acid, solubilized in SDS sample buffer, and equal volumes of each fraction were analyzed by SDS-PAGE. Following transfer to nitrocellulose, proteins were detected with specific antisera to AtELP (Ahmed et al., 1997), AtPEP12p (Conceição et al., 1997), the tonoplast marker pyrophosphatase (Maeshima et al., 1989), and the Golgi marker ARA-4p (Ueda et al., 1996). For quantification of results, blots were digitized on a flat-bed scanner and densitometry was accomplished using NIH Image software. The resulting data for each marker was normalized to their respective backgrounds, and the percent of marker protein detected in each fraction with respect to the total loaded in all fractions was calculated.

Electron Microscopy

Cryosections of *Arabidopsis* roots were done essentially as described previously (Conceição et al., 1997). For immunolabeling, grids were floated on drops in successive solutions at room temperature according to Slot et al. (1991). After blocking in 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS), sections were incubated with primary antibodies to AtPEP12p or AtELP for 4 hr. Excess antibody was removed by multiple washes of 1% BSA in PBS. Primary antibodies were detected by biotinylated goat anti-rabbit IgG for 1 hr, followed by streptavidin-conjugated to 10 nm colloidal gold particles. For double-labeling, the grids were first treated as above for AtPEP12p antibodies, then a second fixation step using 1% glutaraldehyde and 0.02% Gly, followed by a second blocking step with 1% BSA in PBS, was utilized to prevent cross-reactivity of the AtPEP12-antisera in later steps (Slot et al., 1991). The grids were then incubated with either no antisera, or specific antisera for AtELP for 4 hr, followed by a 1 hr incubation with goat anti-rabbit IgG linked directly to 5 nm colloidal gold particles. The grids were washed in distilled water, and stained according to Griffiths et al. (1983). The sections were observed with an Hitachi model H600 electron microscope operating at 75 kHz. All labeling experiments were conducted several times each on independent sections. Quantification of immunogold staining was done by counting the number of gold particles found within a 0.33 μm^2 circle centered on either the Golgi, the cytoplasm, or the nuclear matrix of many independent micrographs.

RESULTS

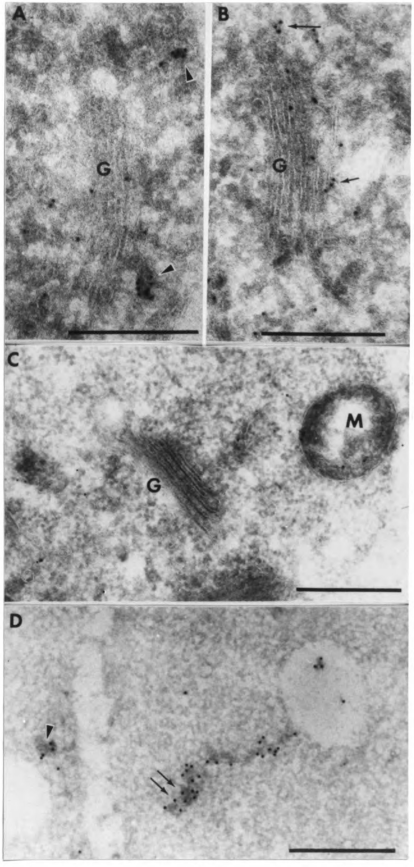
AtELP is localized to the *trans*-Golgi and post-Golgi Membranes

The first step where protein targeting to the vacuole/lysosome differs from secretion occurs in the TGN, where cargo receptors such as the M-6-P receptor of mammalian cells

and CPY receptor (Vps10p) of yeast bind to their specific cargo and redirect it away from the bulk flow of proteins in the secretory pathway (Kornfeld, 1992; Marcusson et al., 1994). Thus, the first step in investigating the role of AtELP as a potential cargo receptor was to examine cryosections of *Arabidopsis* roots for the localization of AtELP.

The majority of the AtELP-associated labeling was found on electron-dense uncoated vesicular structures which were often found near the *trans*-Golgi of the root cells (Figure 3.1A, arrowheads). These structures were similar to the post-Golgi structure previously identified for AtPEP12p in *Arabidopsis* roots (Conceição et al., 1997; and see below). Significant AtELP-labeling was also found to be associated with the Golgi apparatus (Figure 3.1B, small arrows). Statistical analysis of many independent micrographs indicated that the level of Golgi labeling was 9-fold higher than that of the cytoplasm or nuclei; further, almost all of the Golgi-associated labeling was found on the *trans*-side. The orientation of the Golgi was determined based upon appearance and the more electron-dense staining pattern of the *trans*-Golgi. Some lower level of labeling was also found associated with more *cis*- or *medial*-stacks of the Golgi (data not shown), though this may represent AtELP in transit to the *trans*-Golgi. AtELP antisera was not found to label the ER, PM, or tonoplast (data not shown). Labeling with the preimmune sera was not seen upon the Golgi or the post- Golgi structures, and mainly consisted of random background staining (Figure 3.1C). The fixation and cryosectioning conditions used here are designed to maintain antigenicity of membrane proteins, and are not optimal for preservation of coated vesicles, thus, we were unable to directly determine if AtELP was associated with CCVs as has been previously shown biochemically (Ahmed et al., 1997). These results showed that AtELP is localized to the *trans*-face of the Golgi

Figure 3.1
AtELP is localized on the *trans*-Golgi and post-Golgi membranes in cryosections of *Arabidopsis* roots. Sections were treated with antisera to AtELP (A and B), preimmune for AtELP (C), or with antisera to AtPEP12p (D) followed by biotinylated goat anti-rabbit secondary antibodies. Antibodies were then detected by streptavidin conjugated to 10 nm colloidal gold. G, Golgi; M, mitochondria. Bar = 0.5 μm.


A
G
B
G
C
G
M
D

in *Arabidopsis* root cells, as well as being found in other structures which are likely post-Golgi membranes.

We have further investigated the localization of AtPEP12p, and have found that, in addition to the 100 nm electron-dense uncoated vesicles reported earlier (Conceição et al., 1997; Figure 3.1D, arrowhead), AtPEP12p-labeling was also associated with electron-dense reticulo-tubular compartments with diameters of approximately 100 nm (Figure 3.1D, double arrow). These tubules were found to heavily label with AtPEP12p antisera, and occasionally were found to attach to small electron-lucent membranes which are perhaps small vacuoles. Due to the fact that these tubules share the same electron-dense staining pattern, and a similar diameter (100 nm), it is possible that the previously described vesicular structures (Figure 3.1D, arrowhead) represent cross-sections through these tubules. We now refer to both structures as a PVC. No staining of the PVC was seen with preimmune sera (Conceição et al., 1997, and data not shown).

The cytoplasmic tail of AtELP interacts specifically with the TGN-associated AP-1 adaptor complex

Considering that AtELP has been reported to be associated with CCVs (Ahmed et al., 1997), and that its cytoplasmic tail contains a potential Tyr-motif (YMPL, residues 606-609), it was therefore likely that AtELP would interact with the adaptor complex as part of forming CCVs. The recognition of a Tyr-motif by either the AP-1 or AP-2 complex is dependent on the sequence context surrounding the Tyr-motif (Kirchhausen et al., 1997; Rapoport et al., 1998), thus, to determine which type of AP-complex is capable of interacting with the cytoplasmic tail of AtELP, we performed an *in vitro* assay. Unfortunately, although some components of the plant APs have been identified by

sequence homology (Holstein et al., 1994; Maldonado-Mendoza and Nessler, 1996; 1996), they have not been characterized biochemically. So, considering the likely conservation in the AP-mechanisms, we chose to use the well defined mammalian AP complexes. The μ-subunits of both the TGN-associated AP-1 and the PM-associated AP-2 adaptor complex of mammalian cells have been shown to interact with the Tyr-motifs of several mammalian proteins such as Lamp-1 and TGN38 (Rapoport et al., 1997; 1998; Ohno et al., 1995). Recent studies have shown the specificity of AP-1 for the Tyr-motif of Lamp-1 (Rapoport et al., 1998), and the specificity of AP-2 for TGN38 (Rapoport et al., 1997). In these assays, photoactivatable-cross-linking is used to show binding of a biotinylated Lamp-1 or a TGN38 cytoplasmic-tail peptide to the μ-subunit of the AP complexes, which is subsequently detected with streptavidin conjugated to horseradish peroxidase. Using this assay, we addressed whether synthetic peptides corresponding to the cytoplasmic tail of AtELP would compete for (and therefore block) binding of the AP complexes to Lamp-1 or TGN38 peptides.

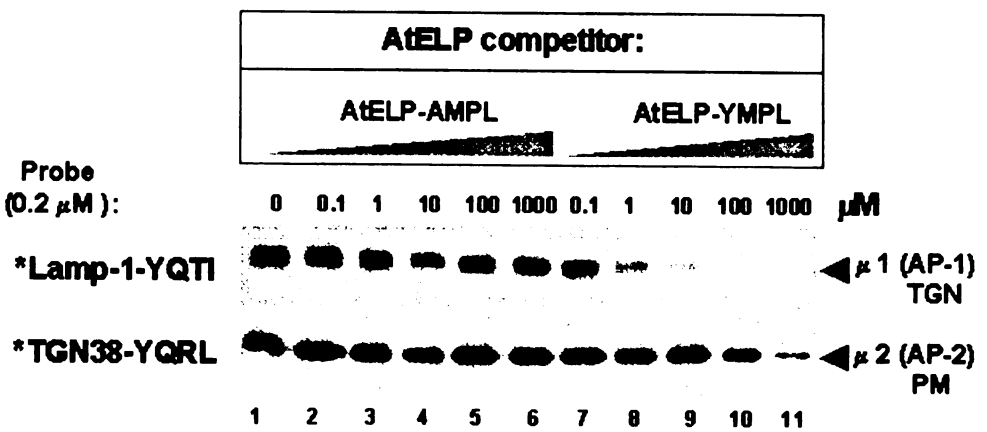
The synthetic peptides used in this study are shown in Figure 3.2A. The biotinylated, photoactivatable *Lamp1-YQTI and *TGN38-YQRL tail peptides have been described previously (Rapoport et al., 1997; 1998). The AtELP peptides correspond to residues 599-to-612 of the cytoplasmic tail (YMPL), or to this sequence with Tyr-606 changed to Ala (AMPL) to potentially inactivate the Tyr-motif. In the absence of AtELP peptides, 100% binding of the Lamp-1 and TGN38 peptides was seen to μ1 of the AP-1 and to μ2 of the AP-2 complexes, respectively (Figure 3.2B, lane 1). The mutated AtELP-AMPL peptide was unable to compete binding of either μ1 or μ2 (Figure 3.2B, lanes 2-6) to *Lamp1-YQTI or *TGN38-YQRL (respectively). One micromolar concentration of the

Figure 3.2
Interaction of the cytoplasmic tail of AtELP with the AP-1 complex. A schematic representation of peptides used for the cross-linking studies involving AP-1 and AP-2 complexes is shown in (A). Complete sequences of the Lamp-1, TGN38 and AtELP cytoplasmic tails containing the Yxxϕ motif are compared with the sequences of the synthetic peptides used in this study. The Tyr-based motifs are highlighted and underlined. The *Lamp1-YQTI and *TGN38-YQRL peptides were modified by replacement of an alanine with the uv-photoactivatable cross-linking agent benzoylphenylalanine (BPA), and by addition of biotin to the N-terminus. AP-1 and AP-2 complexes (0.2 mg/mL) purified from calf brain coated vesicles were incubated with 0.2 μM of either *Lamp1-YQTI (B, upper panel) or *TGN38-YQRL (B, lower panel) cross-linking peptides and increasing amounts (0 to 1000 μM) of the AtELP-YMPL or mutated AtELP-AMPL peptides as competitors. The appearance of the cross-linked photoreactive peptides to μ1 of AP-1 (upper panel) or μ2 of AP-2 (lower panel) upon UV irradiation was tested after separation by SDS-PAGE using streptavidin conjugated to horseradish peroxidase.

A.

	Yxxϕmotif ▼
Lamp-1 (cytoplasmic tail)	RKRSHAG**YQTI**
	biotin BPA
	| |
***YQTI**	KKRSH: G**YQTI**
TGN38 (cytoplasmic tail)	HNKRKIIAFALEGKRSKVTRRPKASD**YQRL**NLKL
	biotin BPA
	| |
***YQRL**	KVTRRPK: SD**YQRL**
AtELP (cytoplasmic tail)	IRSYMDAEIRGIMAQ**YMPL**ESQPPNTSGHHMDI
YMPL	IRGIMAQ**YMPL**ESQ
AMPL	IRGIMAQ**AMPL**ESQ

B.



AtELP-YMPL peptide was sufficient for half-maximal competition, and 10 μM abolished greater than 90% of the binding of μ1 (AP-1) to the *Lamp1-YQTI peptide (Fig 3.2B, upper panel, lanes 7-11), indicating that the cytoplasmic tail of AtELP was likely binding to the AP-1 complex with high-affinity. Only at a 1000-fold higher concentration was the AtELP-YMPL peptide able to compete away binding of μ2 (AP-2) to the *TGN38-YQRL peptide (Figure 3.2B, lower panel, lanes 7-11). Hence, the AtELP peptide appears to have a higher affinity for μ1 of the TGN-specific AP-1 than for μ2 of the PM-specific AP-2 complex. Further, the fact that the AtELP-AMPL peptide was unable to block binding suggests that the Tyr-motif of AtELP is required for interaction with the AP complex. These results, together with the localization of AtELP to the *trans*-Golgi of *Arabidopsis* roots (see above), suggests that AtELP may have a role in CCV-directed trafficking of proteins at the TGN of plant cells.

AtPEP12p and AtELP Partially Cofractionate in Sucrose Density Gradients

Given that AtPEP12p appears to be a t-SNARE residing on a PVC, and that AtELP is a potential vacuolar cargo receptor, it seemed likely that these proteins would at least partially reside on the same membrane population. To determine if this was the case, we subjected *Arabidopsis* root tissue to sucrose density gradients optimized to provide better separation of plant endomembranes (see Methods), and examined the fractionation pattern of AtPEP12p, AtELP, and several available markers for the plant endomembrane system, with antisera specific for each protein.

AtPEP12p can be differentiated into 3 distinct peaks at densities of approximately 1.12, 1.14, and 1.17 g/mL (Figure 3.3A). AtELP was found to have a major low-density peak at 1.08 g/mL, with additional minor peaks found at higher densities of 1.12, 1.14,

and 1.17 g/mL (Figure 3.3B). Importantly, it appeared that AtPEP12p and AtELP cofractionated in the 1.12, 1.14, and 1.17 g/mL peaks, a result that was more clearly seen following quantification of the fractions by densitometry (Figure 3.3C). Neither protein cofractionated with pyrophosphatase (Figure 3.3C), a marker for the tonoplast (Maeshima et al., 1989). In addition, the fractionation pattern of AtPEP12p and AtELP was distinct from ARA-4p (data not shown), a marker for the Golgi (Ueda et al., 1996). The peaks observed for these proteins are unlikely to represent the residual interfaces of the step gradient because: (a) following centrifugation, the density profile of the gradient is virtually linear (Figure 3.3D); and (b) the densities of the peaks observed for these proteins are not consistent with the proteins accumulating at an interface between steps. Thus, the cofractionation of AtELP and AtPEP12p in the higher-density peaks suggests that these two proteins reside on the same compartment, which likely represents a PVC. Meanwhile, the low density peak (1.08 g/mL), which only contained AtELP, may represent the *trans*-Golgi staining seen for AtELP (see Figure 3.1B), or may potentially be a population of vesicles in transit to-or-from the PVC.

AtELP and AtPEP12p colocalize on a PVC

We have shown above, that AtELP was localized to the *trans*-Golgi as well as to electron-dense post-Golgi membranes in cryosections of *Arabidopsis* roots. These electron-dense vesicles were similar to the late post-Golgi organelle (now called a PVC) that contained AtPEP12p (Conceição et al., 1997; Figure 3.1D, arrowhead). Together with the cofractionation of AtELP and AtPEP12p in density gradients, this suggested that AtELP and AtPEP12p would likely colocalize in these structures. To investigate this possibility, we performed double-labeling experiments on cryosections of *Arabidopsis*

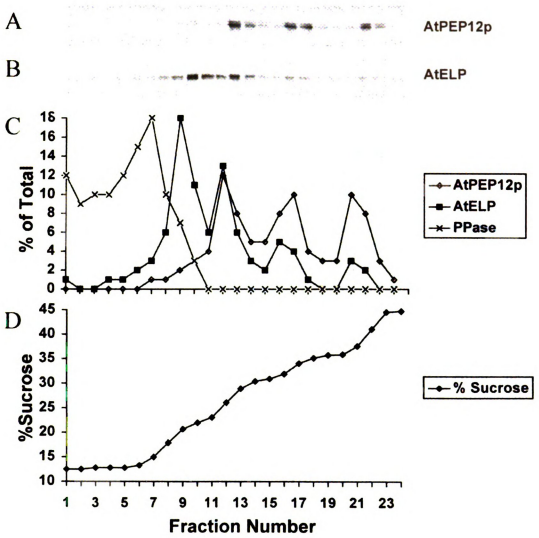


Figure 3.3.
Equilibrium density gradient analysis of *Arabidopsis* roots. Post-nuclear membranes were loaded onto a sucrose step gradient and spun to equilibrium. Twenty-four fractions were collected from the top, trichloroacetic acid precipitated, and equal volumes of each fraction were separated by SDS-PAGE and transferred to nitrocellulose. AtPEP12p (A), AtELP (B), and pyrophosphatase were detected using specific antisera to each protein. Blots were analyzed by densitometry, and the percent of the total marker protein detected in each fraction for AtPEP12p, AtELP, and pyrophosphatase was plotted in (C). The sucrose concentration of each fraction was determined by refractometry, and was plotted in (D).

roots in which AtPEP12p was first labeled with specific antisera, and detected with 10-nm gold. A second fixation and blocking step was then performed prior to incubating the sections with either no antisera or antisera specific to AtELP, followed by detection with 5-nm gold.

We found that both AtPEP12p and AtELP antisera specifically labeled many of the same electron-dense uncoated vesicular structures (Figure 3.4, arrowheads). No labeling of these structures with 5 nm gold was seen in the absence of AtELP antisera (data not shown). Figure 3.4 represents a glancing section through the Golgi apparatus, and thus, it is difficult to clearly identify the *trans*-face. Still, some vesicles were found to only be labeled with AtELP (5 nm gold), and these appeared to be associated with the Golgi (Figure 3.4, small arrow). These results indicated that some population of AtELP coexists with AtPEP12p on the same membrane population, and are consistent with the biochemical cofractionation reported above. Further, considering the likely role of AtELP in trafficking of vacuolar proteins from the TGN, the colocalization of AtELP with AtPEP12p on the electron-dense structures supports our argument that these membranes represent a PVC.

DISCUSSION

The plant vacuole is a vital multifunctional organelle with roles in protein storage, osmoregulation, cell growth, and development (reviewed in Robinson and Hinz, 1997). Trafficking of proteins to the vacuole has been extensively studied, and the signals involved in targeting to the plant vacuole have been well defined (Chrispeels and Raikhel, 1992). Some components of this trafficking machinery have begun to be characterized including putative cargo receptors involved in vacuolar targeting (BP-80,

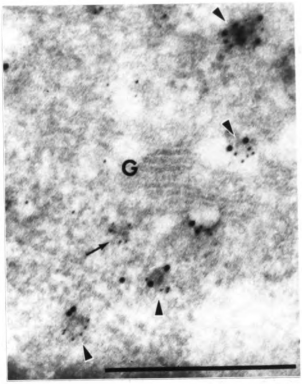


Figure 3.4
AtELP and AtPEP12p colocalize on a PVC in cryosections of *Arabidopsis* roots. Section was incubated with antisera to AtPEP12p, followed by biotinylated goat anti-rabbit secondary antibodies, then visualized with streptavidin conjugated to 10 nm colloidal gold. Following a second fixation step (see Methods), the same sections were incubated with antibodies to AtELP, followed by goat anti-rabbit secondary antibodies conjugated to 5 nm colloidal gold. G, Golgi. Bar = 0.5 μm.

Paris et al., 1997; and AtELP, Ahmed et al., 1997), as well as proteins which are likely to function in vesicle fusion steps during trafficking to the vacuole: the t-SNAREs AtPEP12p (Bassham et al., 1995; Conceição et al., 1997) and AtVAM3p (Sato et al., 1997), and the Sec1p-homologue AtVPS45p (Bassham and Raikhel, 1998). Here, we have reported on further characterization of the plant vacuolar targeting machinery. We have shown that AtELP, consistent with a proposed role as a cargo receptor for vacuolar cargo proteins and its biochemical association with CCVs, is localized at the *trans*-Golgi, and can interact *in vitro* with the proteins of the mammalian TGN-specific AP-1 adaptor complex. Further, we have shown through both biochemical and immunocytochemical means, that a population of AtELP colocalizes with the t-SNARE AtPEP12p on a membrane compartment which is likely to be a PVC.

Our results have shown that the cytoplasmic Tyr-based motif of AtELP can efficiently compete for the mammalian AP-1 with the Tyr-based motif of a protein known to function at the TGN (Lamp-1). It is important to note that AtELP has much less affinity for the mammalian AP-2 complex, which argues against an involvement of AtELP in endocytic CCVs at the plasma membrane. Although a few components of the adaptor complexes have been identified in plants (Holstein et al., 1994; Maldonado-Mendoza and Nessler, 1996; 1996) they have not been well characterized. Moreover, it has not been possible to clearly distinguish whether these components are members of the AP-1 or AP-2 complexes of plant cells. Thus, it is not yet possible to carry out similar experiments to those shown here using the plant adaptor complexes. Regardless, the overall conservation apparent in the adaptor complexes among various cell types suggests that our results using the mammalian adaptor complex are most likely relevant to the

function of AtELP in plants. The immunological localization of AtELP to the *trans*-Golgi, together with the fact that AtELP has been found associated with CCVs (Ahmed et al., 1997), suggest that AtELP may function in CCV-directed trafficking of vacuolar proteins at the TGN of plant cells.

The cofractionation of AtPEP12p and AtELP in sucrose density gradients is supported by the immunogold-electron microscopy. Based upon the unique fractionation pattern of AtPEP12p in density gradients as well as immunoelectron microscopy (Conceição et al., 1997), we have previously suggested that AtPEP12p represents a novel marker for a PVC. The fact that we now find AtELP, a protein possibly involved in transport of vacuolar cargo, associated with AtPEP12p further supports that this organelle represents a PVC in plant cells. It is interesting that the PVC which is described here for AtPEP12p has such a heterogeneous fractionation pattern in density gradients (*i.e.*: three peaks). It is possible that the PVC is a fragile organelle which fragments during the isolation and centrifugation procedures. Although, the consistent fractionation pattern observed from many independent experiments (data not shown) makes this seem unlikely. The heterogeneity could also be explained due to the apparent complexity of the vacuolar system in plant cells. At least four biochemically distinct pathways for targeting of soluble and membrane proteins to the plant vacuole have been described (reviewed in Bassham and Raikhel, 1997). Moreover, some plant cells have been reported to have two types of vacuoles, which each receive different cargo proteins (Hohl et al., 1996; Paris et al., 1996). Thus, it is possible that AtPEP12p resides on many separable PVCs as part of its role in receiving protein traffic.

It is also intriguing to speculate that the low-density (1.08 g/mL) peak of AtELP, which does not appear to cofractionate with AtPEP12p, may represent a biochemical marker for the TGN in plant cells. A result that is supported by the electron microscopic studies which found only AtELP associated with the *trans*-Golgi, while AtELP and AtPEP12p appear to consistently colocalize on PVCs. Although we find some AtELP-labeling on the Golgi by electron microscopy, our results have shown that AtELP does not cofractionate with the Golgi marker ARA-4p; nor did AtELP cofractionate with the Golgi-associated enzymatic activity of fucosyl transferase (Ahmed et al., 1997). This may suggest that AtELP passes quickly through the Golgi, and cannot be found there at steady state (*i.e.*: in density gradients). Alternately, the *trans*-Golgi membranes stained by AtELP may represent a biochemically-distinct membrane from the Golgi-proper. Or, it may also be that ARA-4p and fucosyl transferase are restricted to the *cis*-side of the Golgi in roots, and thus, sediment in the gradient as a distinct population of membranes. Clearly, a more thorough examination of the biochemical markers for the plant Golgi is required. It is also possible that this low-density peak may represent vesicles in transit to-or-from the PVC which contain AtELP. Further experiments are needed to clearly identify the nature of this vesicle population.

Our data argues for a role for AtELP in transporting cargo from the TGN to the PVC; yet, the nature of the cargo that AtELP may be carrying remains unclear. AtELP is clearly homologous to BP-80 (72% identical), a protein shown to associate with the N-terminal VSS of some soluble vacuolar targeted proteins (Kirsch et al., 1994). However, an AtELP/BP-80 homolog has recently been characterized from pumpkin (PV72/82) that binds *in vitro* to both a C-terminal and internal, but not N-terminal VSS from pumpkin

pro2S albumin (Shimada et al., 1997). We have not been able to show an *in vitro* association of AtELP with either the N-terminal VSS of sweet potato sporamin or the C-terminal VSS of barley lectin (Ahmed, S. U., Bar-Peled, M., and Raikhel, N. V., unpublished observations), though this is perhaps due to the lack of posttranslational modification and improper folding of the bacterially-expressed protein used in these studies. Together with the fact that there are several *AtELP*-like genes found in the *Arabidopsis* EST database (Ahmed et al., 1997), as well as two full-length pea *BP-80*-like clones identified by Paris et al. (1997), this suggests that AtELP, pea BP-80 and pumpkin PV72/82 may be members of a family of related vacuolar sorting receptors in plant cells. The fact that each may have different affinities for each type of VSS may provide the specificity required for the sorting of multiple proteins to the vacuole by different pathways.

In conclusion, we have further examined the components of the vacuolar targeting machinery in plants. As outlined above, our findings suggest that AtELP is involved in CCV-directed trafficking of cargo proteins from the TGN of plant cells to the PVC defined by AtPEP12p. The exact nature of this prevacuolar organelle is still unclear due to the complexity of the vacuolar pathway in plant cells. However, the connection between these two markers, as well as to other endomembrane markers which we continue to characterize, will allow us to investigate this complexity in greater detail.

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Chapter 4

Functional Analysis of AtELP in *Arabidopsis thaliana*

ABSTRACT

Clathrin-coated vesicles are known to mediate protein transport through the secretory pathway in plant cells. Some soluble vacuolar proteins are thought to be bound by transmembrane cargo receptors at the *trans*-Golgi network that direct these proteins into clathrin-coated vesicles. We have previously characterized a putative vacuolar sorting receptor AtELP from *Arabidopsis*, which is localized at the *trans*-Golgi, a prevacuolar compartment and is associated with clathrin-coated vesicles in plants. Here, we show that AtELP binds to the amino-terminal targeting peptides (NTPP) of barley proaleurain and sweet potato prosporamin, in a pH dependent manner. The putative receptor does not bind to the carboxy-terminal targeting peptides (CTPP) of probarley lectin or protobacco chitinase. The binding of AtELP to the aleurain and sporamin determinants is dependent on the 'NPIR' targeting motif previously found to be important for proper *in vivo* sorting of these cargo proteins to the plant vacuole. These biochemical results are supported by electron microscopy studies which reveals the colocalization of AtELP with the vacuolar cargo protein NTPP-sporamin at the Golgi apparatus and structures near the vacuole, but not with the barley lectin-CTPP cargo protein in roots of transgenic *Arabidopsis* plants. Taken together, these results strongly suggests that AtELP may function as a vacuolar sorting receptor involved in the targeting of NTPP-containing proteins in *Arabidopsis*.

INTRODUCTION

Unlike yeast and mammalian vacuoles/lysosomes, the plant vacuole may serve as a lytic compartment for the degradation of materials as well as store proteins. However, the stored proteins must be kept separate from proteases that would degrade them until necessary. In support of this requirement for a physical separation, there is a growing evidence now for the presence of multiple vacuoles in the same cell of some plant tissue. Moreover, there appears to be several different routes involved in the intracellular trafficking of vacuolar proteins, some of whose features may be unique to plants (reviewed in Chapter 1). Clearly, the presence of many different routes for protein transport to multiple plant vacuoles suggests that there are several mechanisms associated with them, indicating the complex nature of plant vacuolar protein transport.

Soluble proteins destined for the vacuole in plants first enter the endoplasmic reticulum (ER) from which they are transported to the Golgi apparatus. Upon reaching the TGN, these vacuolar proteins are specifically sorted by virtue of vacuolar-sorting signals (VSSs), into transport vesicles that bud from the TGN, and are then thought to travel to and fuse with a prevacular compartment (PVC) where the cargo is delivered. The proper delivery of the proteins therefore depends on the VSSs and the transport machinery that interacts with this information. Considerable progress has been made so far in understanding the sorting signals on soluble plant vacuolar proteins (reviewed in Vitale and Raikhel, 1999). The deletion of these signals results in the secretion of the vacuolar proteins. Conversely, fusion of the VSSs to otherwise secreted proteins can redirect the reporter proteins to the vacuole.

To further our understanding of the vacuolar protein transport pathways in plants, we have recently isolated and characterized several components of the cellular transport machinery from *Arabidopsis thaliana* (reviewed in Chapter 1). In particular, we have isolated and characterized AtELP, a putative vacuolar sorting receptor that shares many features common to several eukaryotic sorting receptors. Its biochemical properties and subcellular distribution suggests that AtELP may play a role in targeting of proteins to the plant vacuole (Chapter 1 and 2). Here, we have investigated further the role of AtELP in vacuolar targeting using a biochemical assay and have found that it interacts with the NH_2-terminal propetide (NTPP)-containing vacuolar sorting signals of barley aleurain (Aleu) and sweet potato sporamin (Spo), but not with the COOH-terminal (CTPP)-sorting signals of barley lectin (BL) or tobacco chitinase (TobChit) in a sequence specific and pH dependent manner. Moreover, using immunoelectron microscopy, we have found that in transgenic *Arabidopsis* plants, AtELP coloclalizes with the vacuolar cargo reporter protein NTPP-Spo, but not with the BL-CTPP cargo protein. These results are consistent with the proposed function of AtELP as a receptor that would select proteins in the *trans*-Golgi for sorting to CCVs and delivery to the vacuole in Arabidopsis.

MATERIALS AND METHODS

Affinity Column Chromatography

To prepare affinity columns, peptides were commercially synthesized at Research Genetics Inc., Huntsville, AL to >85% purity. For the NTPP-peptides, a cysteine residue was added at the carboxy-terminal end of each peptide for subsequent chemical coupling to "Sulfolink" agarose beads (Pierce) according to the manufacturer's protocols. The BL-CTPP-peptide was coupled to Affigel -15 (BioRad) beads according to the

manufacturer's protocols. The proaleurain, prosporamin, probarley lectin and protobacco chitinase propeptides contained sequences previously shown to be essential for the proper targeting of each of the vacuolar proteins (reviewed in Vitale and Raikhel, 1999). Affinity columns were prepared with 3 mg of each of the NTPP peptides by immobilizing them to coupling gels. *Arabidopsis* cell suspension cultures were grown as previously described (Ahmed et al., 1997). Up to 1 liter of cells were collected for each experiment 5-7 days after subculture and harvested for the preparation of total microsomes, which were prepared as follows: Cells were lysed in 300 ml of 0.1 M MES-NaOH, pH 6.5, 0.3 M Sorbitol, 1 mM EGTA, 0.5mM $MgCl_2$, 0.02% NaN_3, 1mM PMSF, 1mM DTT and 1mg/mL trypsin inhibitor in a commercial blender at 4°C. The total homogenate was passed through two layers of Miracloth (Calbiochem) and centrifuged at 3000*g* for 20 min at 4°C. The resulting supernatant was centrifuged at 20,000*g* for 30 min at 4°C. The supernatant from this step was then collected and centrifuged at 120,000*g* for 1hr at 4°C. The resulting pellet was collected and resuspended in 4-5 mL of "Binding Buffer" (20mM Hepes-NaOH, pH 7.0/150mM NaCl/1mM $MgCl_2$/1mM $CaCl_2$,1mM PMSF) using a dounce homogenizer. To extract integral membrane proteins from the microsome fractions, "CHAPS" was then added to the resuspended microsomes to a final concentration of 1% (v/v) and then incubated by mixing on a rocker at 4°C for 30 min, followed by centrifugation at 120,000*g* for 30 min at 4°C in a TLA 45 rotor, using a Beckman mini-ultracentrifuge to remove the insoluble material. 2 mL of the resulting supernatant (containing ~15-20 mg of detergent extracted proteins) was used as a crude source of AtELP and added to each of the affinity columns. Proteins were bound to the peptide column at room temperature for 1 hr in the "Binding Buffer" and then washed

with 6 column volumes of the same buffer according to previously described protocols (Kirsch et al., 1994; 1996). The bound proteins were then eluted with an "Elution Buffer" (25mM NaOAc, pH 4.0/150mM NaCl/1mM EGTA/1%Triton X-100). Approximately, 800 μL fractions from each of the flow through, washes and the elutions were collected, of which 100 μL of each fraction was analyzed by SDS-PAGE, followed by immunoblotting with anti-AtELP antibodies as described in Ahmed et al (1997). For the competition studies, the bound proteins were eluted by the addition of 3 column volumes of 1-1000 μM of each of the peptide in the "Binding Buffer".

Plant Material

Arabidopsis ecotype Columbia plants were transformed with either the full length BL-CTPP cDNA clone (Dombrowski et al., 1994) or the full length NTPP-Spo cDNA clone (Matsuoka et al., 1995) in the pGA 643 binary vector under the transcriptional control of the CaMV 35S promoter. The transformation was carried out with *Agrobacterium tumefaciens* strain GV3101 using vacuum infiltration as described by Bent et al (1994). Transformants were selected by Kanamycin and the presence of NTPP-Spo and BL-CTPP was detected in several independent lines by protein gel blot analysis using anti-Spo or ant-BL antiserum as described in Matsuoka et al., 1995 and Dombrowski et al., 1993.

Electron Microscopy

The procedures used to prepare grids for thin plastic sections followed by immunolabeling with the appropriate antibodies were essentially as described in Zheng et al., 1999 with some minor modifications. The root tips of 7-10 day-old *Arabidopsis* plants transformed with either Spo or BL were fixed in a 0.05 M sodium phosphate buffer

(pH 7.4) containing 2% formaldehyde and 0.1% glutaraldehyde overnight at 4°C. The specimens were then processed for final fixation and grids containing ultrathin sections (70-80 nm) were prepared according to Zheng et al., 1999. For double-immunolabeling, the grids were first treated with rabbit antiserum specific for AtELP for 4 h at room temperature, followed by 1 hr incubation with biotinylated goat anti-rabbit IgG and then by streptavidin conjugated to 5- or 10-nm gold particles as indicated in the figures. Before labeling with the second antibody, the grids were fixed in 0.1% glutaraldehyde, followed by a second blocking with 2% BSA in PBS-1% Tween-20 to prevent cross-reactivity of AtELP antibodies in later steps (Slot et al., 1991). The grids were then incubated with specific rabbit antiserum for Spo or for BL for 1.5 h at room temperature, followed by 1-h incubation with protein-A conjugated to 10- or 15-nm colloidal gold particles. The control sections were treated with the AtELP, Spo, or BL pre-immune serum as well and showed almost no background (data not shown). The grids were then processed further and several independent sections were examined several times according to the procedure described by Zheng et al., 1999.

To establish the distribution pattern of AtELP, sporamin and barley lectin, areas of the grids were selected that contained cells exhibiting a good ultrastructure. In the quantitative analysis, all membrane structures that were found in close vicinity with and at the Golgi and the vacuole were considered. The Golgi stack itself was recognized by its characteristic morphology of stacked cisternae. In total, approximately 180-220 gold particles were counted for each of the labeled antibodies over 25-30 independent Golgi apparatus that were analyzed in three independent counting sessions. 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 sporamin or barley lectin in the Golgi and other structures near the vacuole (Table 4.1 and 4.2).

RESULTS

AtELP interacts with different amino-terminal vacuolar targeting signals in a sequence-specific and pH-dependent manner.

Our previous molecular, biochemical and electron microscopic studies have indicated that AtELP is a potential vacuolar sorting receptor in Arabidopsis (Ahmed et al., 1997, Sanderfoot et al., 1998). In this report, we investigated the ability of AtELP to interact *in vitro* with peptides representing the targeting determinants of several plant vacuolar proteins. A binding assay for AtELP was performed using affinity chromatography columns containing peptides corresponding to various vacuolar sorting signals. Total detergent extracted membrane proteins from microsomes were bound to each peptide column at pH 7.0 and eluted at pH 4.0. Previous studies with BP-80, a potential vacuolar receptor isolated from pea, indicated that it specifically binds to the NH_2-terminal targeting determinants at neutral pH but not at acidic pH (Kirsch et al., 1994). This approach was therefore used here, based on the hypothesis that *in vivo*, similar to the Man-6-P receptors in mammalian cells (reviewed in Kornfeld, 1992), AtELP/BP-80 binds its cargo ligand at a neutral pH that is characteristic of the Golgi apparatus, while dissociating from it at acidic pH that could be characteristic of a PVC.

The peptides used in this assay correspond to the sequences previously identified in the propeptides that function as VSSs in the transport of each of the vacuolar protein, as indicated in Figure 4.1A. For this binding assay, total microsomes were prepared from *Arabidopsis* cell suspension culture, membrane proteins were extracted in a neutral pH-

buffer containing the detergent "CHAPS" and loaded on to respective columns. The extract was allowed to bind to each column for 1 hr at room temperature and then washed extensively with the same buffer. The proteins retained on the affinity columns were then eluted with an acidic buffer at pH 4. The unbound proteins, those that eluted during the washes, and those that were eluted by the low pH buffer were all analyzed by SDS-PAGE, followed by immunoblotting with anti-AtELP antibodies. These results are shown in Figure 4.1B. Although some AtELP was found to be present in the flow through as well as the first two washes, the majority of the protein was retained on the wild type NTPP-Aleurain (Wt-NTPP-Aleu) and wild type NTPP-Sporamin (Wt-NTPP-Spo) affinity columns and could be subsequently eluted with the acidic buffer. The peptides used to construct these affinity columns represent the Aleu and the Spo sorting signals. In contrast, AtELP was not retained by the mutant NTPP-Aleurain (Mt-NTPP-Aleu) and NTPP-Sporamin (Mt-NTPP-Spo) affinity columns, where the Ile in the NPIR motif present in the Aleu and Spo targeting sequences were changed to a Gly. AtELP was found in the flow-through (FT), the first wash, and no detectable levels of AtELP could be eluted with the acidic buffer (Figure 4.1B). *In vivo*, either the deletion of the NPIRL sequence or the substitution of Gly for Ile in the pro-Spo vacuolar sorting signal results in complete secretion of Spo to the culture media (Nakamura and Matsuoka, 1993). In pro-Aleu, separate contiguous determinants in the NTPP, one of which contains the NPIRL sequence contribute to the maximum level of the vacuolar transport of Aleu (Holwerda et al., 1992). An affinity column representing the barley lectin carboxy-terminal propeptide (BL-CTPP), did not retain any detectable amounts of AtELP from the Arabidopsis microsomal "CHAPS" extract suggesting that AtELP does not interact with the CTPP-

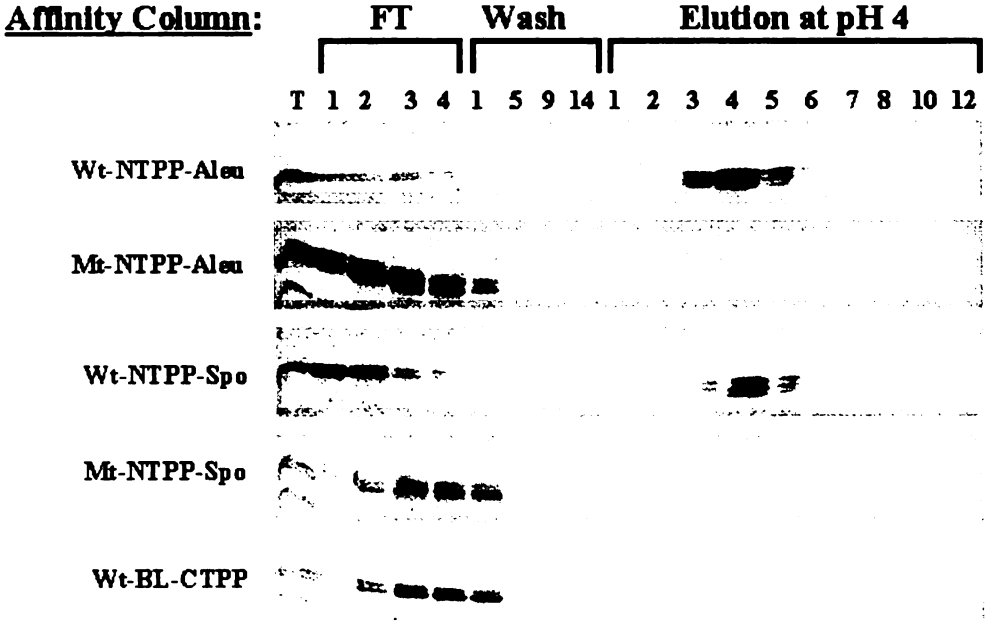
Figure 4.1
Binding of AtELP to vacuolar targeting signals is pH-dependent.
A. The wild-type (Wt) and mutant (Mt) peptide sequences used in this study are shown. These sequences correspond to either the NH_2-terminal (NTPP) or COOH-terminal (CTPP) vacuolar targeting propeptides from each of the indicated vacuolar protein.
B. SDS-PAGE and Western analysis of AtELP binding to the Wt-NTPP-targeting propeptides of aleurain (Aleu) and sporamin (Spo). Binding assay with peptide affinity columns: a detergent ("CHAPS") extract was prepared from total microsomes of *Arabidopsis* suspension cells as described in Materials and Methods and bound to each of the affinity column as indicated for 1 hr at pH 7. Fractions were collected for T, Total "CHAPS" extract that was loaded on to each affinity column; FT, Flow through; Wash, washes with 6 column volumes of binding buffer; Elution, protein eluted under low pH (4.0) condition, and the indicated fraction numbers were analyzed by immunoblotting with anti-AtELP anitsera.

A.

Propeptide	Sequence	Sufficient*	References
Barley aleurain			
Wt-NTPP-Aleu	SSSFADS**NPIR**PVTDRAASTYC[1]	Yes	Howeldra et al., 1992
Mt-NTPP-Aleu	SSSFADS**NPG**RPVTDRAASTYC[1]	N/T*	
Sweet potato sporamin			
Wt-NTPP-Spo	SRF**NPIR**LPTC[1]	Yes	Matsuoka et al., 1993
Mt-NTPP-Spo	SRF**NPG**RLPTC[1]	No	Matsuoka et al., 1993
Barley Lectin			
Wt-BL-CTPP	VFAEAIAANSTLVAE	Yes	Vitale and Raikhel, 1999
Tobacco Chitinase			
Wt-TobChit-CTPP	GLLVDTM	Yes	Neuhaus and Rogers, 1998

[1]A cysteine residue was added to each of the indicated peptide 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 aleurain peptide.
#Indicates whether the propeptide can either redirect a reporter or secreted protein to the vacuole.

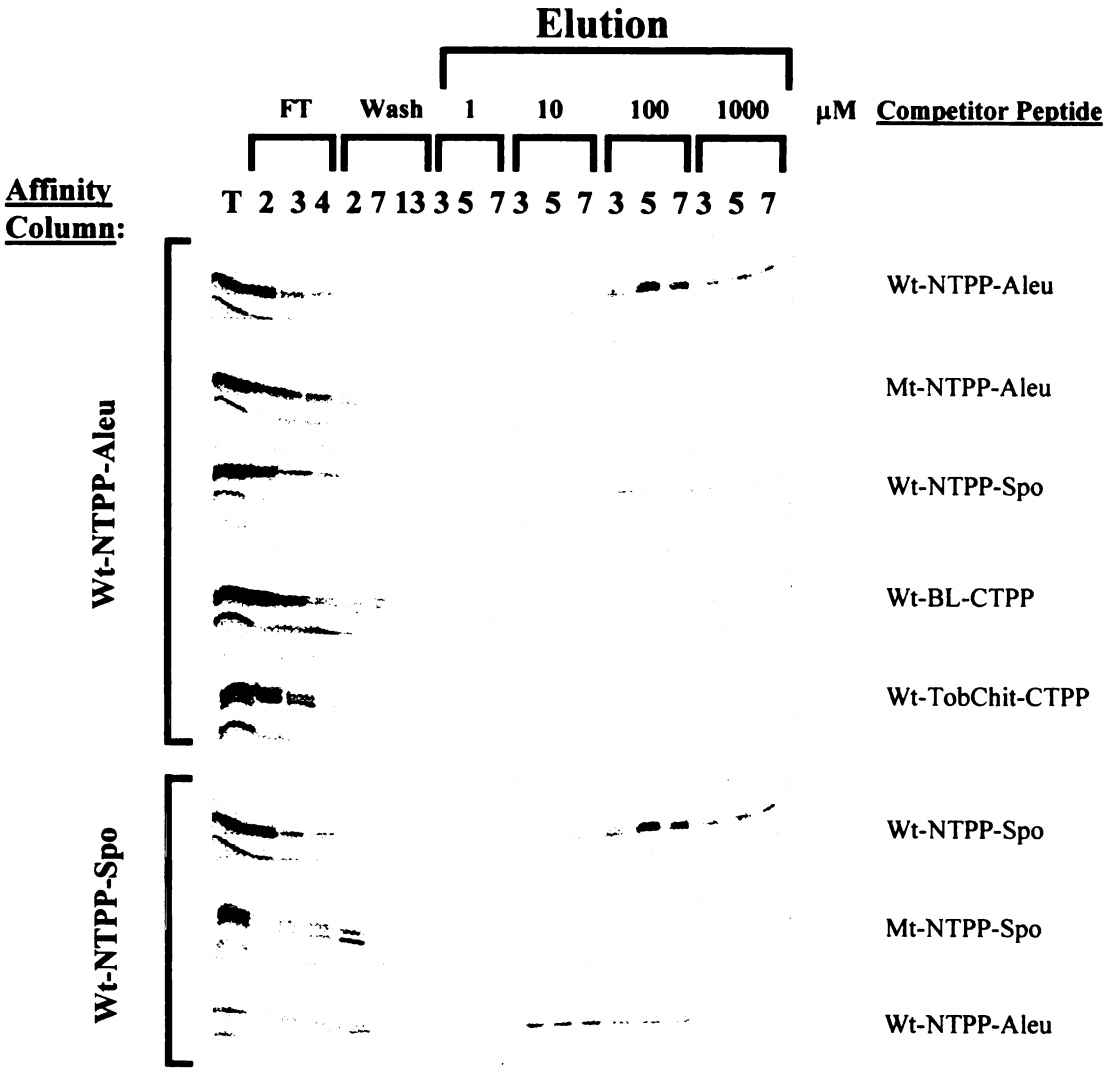
B.



sorting signal of BL.

To further investigate the specificity of AtELP's interaction with Aleu and Spo NTPPs, we carried out competition studies using six different peptides. The "CHAPS" extract was first bound to either the Wt-NTPP-Aleu or Wt-NTPP-Spo affinity columns and washed extensively as described above. The bound proteins were then eluted sequentially by adding 1-1000 μM of each of the following peptides separately at neutral pH: Wt-NTPP-Aleu, Mt-NTPP-Aleu, Wt-NTPP-Spo, Mt-NTPP-Spo, Wt-BL-CTPP and Wt-TobChit-CTPP. These results are described in Figure 4.2. The Wt-NTPP-Aleu and Wt-NTPP-Spo peptides could successfully compete for the binding of AtELP to the corresponding Wt-NTPP-Aleu or Wt-NTPP-Spo peptide columns at ~100 μM concentration. In addition, the Wt-NTPP-Aleu peptide competed for binding to the Wt-NTPP-Spo peptide column, suggesting that these peptides compete for the same binding site. However, AtELP appeared to have a ~10-fold higher affinity for the Wt-NTPP-Aleu peptide than for the Wt-NTPP-Spo peptide. In the presence of 10 μM concentration of the Wt-NTPP-Aleu peptide, the majority of AtELP retained on the Wt-NTPP-Spo affinity column was eluted from the column. In contrast, a 10-fold higher concentration (100 μM) of the Wt-NTPP-Spo peptide was required to elute a similar amount of AtELP retained on the Wt-NTPP-Aleu affinity column. The Mt-NTPP-Aleu or the Mt-NTPP-Spo peptides did not compete for binding. Moreover, up to 1000 μM of peptides corresponding to the vacuolar targeting sequences of the CTPP-barley lectin (BL-CTPP) or CTPP-Tobacco Chitinase (TobChit-CTPP) did not compete for binding. In immunoblot analysis of all our binding experiments, using AtELP antibodies, we constantly detected three closely migrating polypeptides that bound to the Wt-NTPP-

Figure 4.2
Binding of AtELP to the NH2-terminal propeptides is sequence-specific.
The specificity of AtELP binding to the Wt-NTPP-Aleu and Wt-NTPP-Spo propeptides was determined in a competition assay. The "CHAPS" extract was bound to either the Wt-NTPP-Aleu or Wt-NTPP-Spo affinity column as described in Figure 4.1B at neutral pH and the columns were washed extensively. The bound proteins were eluted using increasing concentrations (1-1000 μM) of the indicated peptides as competitors in the same buffer at pH 7. For each of the flow through (FT), wash, and elution steps, fractions were collected, and the indicated fraction numbers were analyzed by immunoblotting with anti-AtELP antisera as in Figure 4.1B.


Elution
FT
Wash
1
10
100
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Competitor Peptide
Affinity Column:
T 2 3 4 2 7 13 3 5 7 3 5 7 3 5 7 3 5 7
Wt-NTPP-Aleu
Wt-NTPP-Aleu
Mt-NTPP-Aleu
Wt-NTPP-Spo
Wt-BL-CTPP
Wt-TobChit-CTPP
Wt-NTPP-Spo
Wt-NTPP-Spo
Mt-NTPP-Spo
Wt-NTPP-Aleu

Aleu and Wt-NTPP-Spo affinity columns. Similarly, these three polypeptides demonstrated equal affinity for the two different NTPPs in the competition assays. The three polypeptides could represent different postranslationl modifications of AtELP, or different isoforms of AtELP that are immunologically related proteins having similar biochemical properties as AtELP. Moreover, these three polypeptides have been previously shown to have identical tissue and subcellular distribution (Ahmed et al., 1997). The results obtained from our binding studies indicate that AtELP interacts with the two NTPP-containing, but not with the CTPP- containing sorting signals, *in vitro*, in a pH dependent manner. In addition, its interactions with these signals are dependent upon the NPIR motif present in the peptides, which is necessary *in vivo* for their proper targeting to the plant vacuole.

AtELP colocalizes with the vacuolar cargo protein NTPP-Spo, but not with BL-CTPP in transgenic *Arabidopsis* roots

Based on the binding experiments described above, AtELP may serve as a vacuolar sorting receptor in Arabidopsis for NTPP-containing proteins. To investigate this possibility *in vivo*, electron microscopy was used to carry out double-labeling experiments on ultra-thin root sections of transgenic Arabidopsis plants expressing the vacuolar cargo reporter proteins NTPP-Spo or BL-CTPP. These two reporter proteins have been previously shown to be transported by distinct pathways (Matsuoka et al., 1995). Sections were first labeled with anti-AtELP antiserum and detected with 5- or 10-nm gold. Subsequent to a second fixation and blocking step, the sections were incubated with either anti-NTPP-Spo or anti-BL-CTPP antisera, followed by detection with 10- or 15-nm gold particles. Representative photomicrographs of root sections prepared from

transgenic NTPP-Spo or BL-CTPP plants are shown in Figure 4.3 and 4.4. In these sections, AtELP was found to colocalize with the vacuolar cargo protein NTPP-Spo at the *trans*-Golgi and in structures near the vacuole (Fig. 4.3 A-C), while the majority of NTPP-Spo antiserum labeled the vacuole (Fig. 4.3D, Table 4.1). However, in sections prepared from plants transformed with transgenic BL-CTPP, although AtELP and BL-CTPP were found to label the same Golgi apparatus, they clearly localize to different parts of the Golgi cisternae. No colocalization of AtELP and BL-CTPP were observed (Fig. 4.4A-C). Again, the majority of the BL-CTPP antiserum labeled the vacuole (Fig. 4.4D, Table 4.2). Quantitative analysis of the AtELP and sporamin colocalization revealed that 74% of the AtELP labeled gold particles colocalized with 56% of the sporamin labeled gold particles in the Golgi and structures near the vacuole (Table 4.1). Colocalization is defined here as the occurrence of two or more gold particles labeled with AtELP and sporamin with a distance of 30-50 nm. Similar analysis of the AtELP and barley lectin localization studies revealed no colocalization of the two proteins in any of the micrographs investigated (Table 4.2). These double-immunogold labeling studies complement the results obtained from the *in vitro* assays described above, and taken together these data strongly suggest that AtELP serves as a vacuolar sorting receptor in Arabidopsis cells for proteins targeted by NH_2-terminal propeptides.

DISCUSSION

Most vacuolar proteins are sorted away from those that are secreted at the TGN, a process that requires the presence of specific sorting signals within the structure of these proteins. Similar to yeast and mammalian cells, the transport of proteins to the plant vacuole has been found to be saturable, indicating the involvement of sorting receptors

Figure 4.3
AtELP colocalizes with NTPP-Sporamin in transgenic *Arabidopsis* root tissues.
In double immunogold labeling experiments of AtELP and sporamin, thin sections of transgenic *Arabidopsis* roots expressing NTPP-Spo were first treated with anti-AtELP antisera, and bound antibody was visualized with biotinylated goat anti-rabbit IgG and then by streptavidin conjugated to 10-nm gold. After a second fixation step and blocking with an excess of BSA, the section was treated with anti-sporamin serum and the bound antibody was visualized with protein-A conjugated to 15-nm colloidal gold. Arrows indicate the 15-nm labeled anti-sporamin antibody and arrowheads the 10-nm labeled anti-AtELP antibody. Three different sections are shown in plates A-C, revealing colocalization of AtELP and sporamin in the Golgi (G), and structures near the vacuole (V) and the predominant staining of sporamin in the vacuole (D) Control experiments using 10-nm labeled anti-AtELP antibodies and 15-nm labeled pre-immune serum for sporamin antibodies (E) and 15-nm labeled anti-sporamin antibodies and 10-nm labeled pre-immune serum for AtELP antibodies (F). Bars represent 0.1 μm.

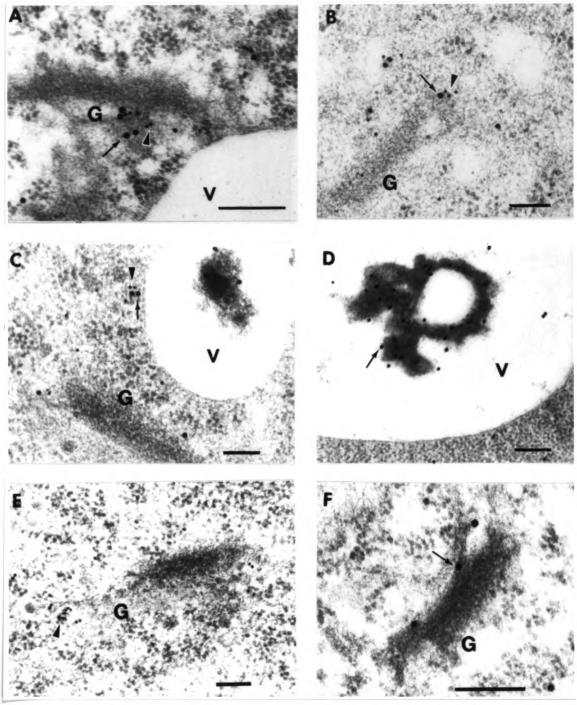
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Figure 4.4
AtELP does not colocalize with barley lectin in transgenic *Arabidopsis* roots.
In double immunogold labeling experiments of AtELP and BL-CTPP, thin sections of transgenic *Arabidopsis* roots expressing BL-CTPP were treated with anti-AtELP antisera, and bound antibody was visualized with biotinylated goat anti-rabbit IgG and then by streptavidin conjugated to 10-nm gold. After a second fixation step and blocking with an excess of BSA, the section was treated with anti-BL serum and the bound antibody was visualized with protein-A conjugated to 15-nm colloidal gold. Arrows indicated the 15-nm labeled anti-BL antibody and arrowheads the 10-nm labeled anti-AtELP antibody. Three different sections are shown in plates A-C, revealing staining of AtELP and/or barley lectin in vacuole (V) and Golgi. The predominant staining of barley lectin is found in the vacuole (D) Control experiments using 10-nm labeled anti-AtELP antibodies and 15-nm labeled pre-immune serum for barley lectin antibodies (E) and 15-nm labeled anti-barley lectin antibodies and 10-nm labeled pre-immune serum for AtELP antibodies (F). Bars represent 0.1 μm.

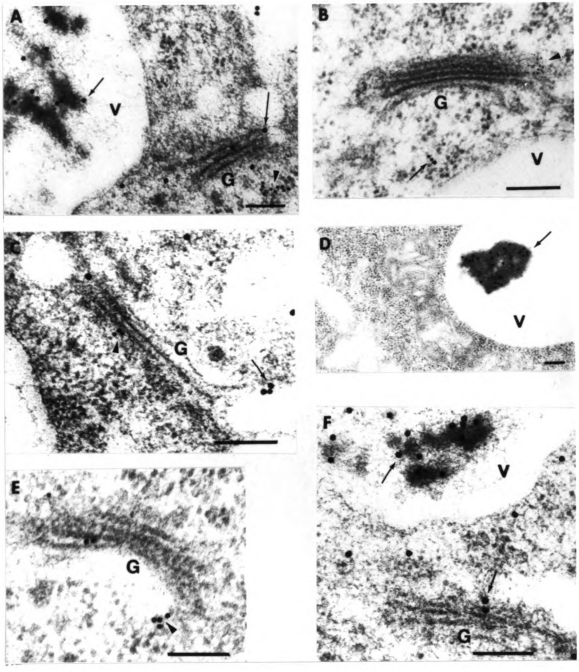
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Table 4.1 Relative distribution of AtELP and Sporamin over intracellular compartments in transgenic Arabidopsis roots

	Golgi	Vacuole	Other Structures	Colocalization
AtELP	73	0	27	74
Sporamin	20	70	10	56

Table 4.2 Relative distribution of AtELP and Barley Lectin over intracellular compartments in transgenic Arabidopsis roots

	Golgi	Vacuole	Other Structures	Colocalization
AtELP	82	0	18	0
Barley Lectin	12	80	8	0

Numbers represent the mean percentages of total gold particles found over the indicated compartments over three independent experiments. The percent of AtELP-specific gold particles that colocaize with the Sporamin- or Barley Lectin-specific gold particles within each Golgi stack and other structures is also shown. Majority of the labeling for Sporamin and Barley Lectin were found in the vacuole.

that might interact with these signals at the TGN (reviewed Vitale and Raikhel, 1995). In mammalian cells, phosphomannosyl residues attached to soluble proteins destined for the lysosome serve as targeting signals to direct sorting away from the proteins destined for secretion. The mannose-6-phosphate receptors (M-6-PRs) at the TGN bind with high affinity to these post-translationally added sugar modifications, while the cytoplasmic tail of these receptors direct the receptor-ligand complexes into CCVs that are directed to the endosome. Upon delivery of the cargo protein in the endosome, the receptor is recycled back to the TGN for further rounds of sorting (reviewed in Kornfeld, 1992). In yeast, similar to that in plants, targeting information for vacuolar transport of soluble proteins have been found to reside in short stretches of amino acids that are part of the unprocessed forms of the proteins. So far the vacuolar sorting receptor, Vps10p, has been found to sort several different vacuolar proteins by cycling between a late Golgi compartment and the prevacuolar compartment (Marcusson et al., 1994; Cooper and Stevens, 1996).

In plants, we have previously characterized a sorting receptor-like protein, AtELP, from Arabidopsis whose biochemical properties and subcellular location suggest that it is a candidate for a vacuolar sorting receptor (Chapter 2 and 3). In this report we show that AtELP specifically interacts with the NTPP-sorting signals of aleurain and sporamin. Moreover, the binding of AtELP to the NTPPs involve the NPIR motif, which was found to be functionally important in prosporamin propeptide *in vivo* (Matsuoka and Nakamura, 1991). The ability of different peptides to compete for binding with the peptides corresponding to the aleurain or sporamin NTTPs parallels the known ability of the different NTPP-sorting signals to direct vacuolar targeting (Matsuoka and Nakamura,

1991; Holwerda et al., 1992).

In competition experiments reported here, the Wt-NTPP-Aleu peptide competed ~10-fold more strongly than the Wt-NTPP-Spo peptide for binding to AtELP. Similar observation was 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 here. Both the NTPPs contain the characteristic NPIR motif, which is required for the *in vitro* binding of AtELP. However, the *in vivo* functional importance of this motif has been demonstrated only for sporamin (Matsuoka and Nakamura, 1991). More recently, the isoleucine (I) residue in this motif has been found to be critical for efficient *in vivo* targeting of sporamin (Matsuoka and Nakamura, 1999). This isoleucine can only be substituted with a valine at the same position without significantly altering sporamin targeting to the vacuole. In contrast, it has been previously shown that efficient vacuolar targeting of aleurain requires the presence of three separate contiguous determinants within the Wt-NTPP-Aleu sorting signal, one of which contains the NPIR motif (Holwerda et al., 1992). However, the presence of only the NPIR motif as the sorting signal is capable of targeting only 3-7% of the protein to the vacuole. This indicates that the binding of AtELP to the aleurain sorting signal in vivo may not solely depend on the NPIR motif, although the isoleucine residue in the motif appears to be required for in vitro interactions. It is also possible that *in vivo* binding of AtELP to the sorting signals may not depend exclusively on the binding motifs, but also on the context in which these motifs are presented. Alternatively, there may be different domains within AtELP that is capable of differentially interacting with multiple signals. In this connection, Vps10p, a

sorting receptor for the yeast carboxypeptidase Y (CPY), has been recently shown to contain multiple sites for binding several vacuolar proteins (Jorgensen et al., 1999). This could also explain some of variations observed in the *in vitro* binding properties of BP-80.

Our *in vitro* binding studies also show that the CTPPs of barley lectin (Wt-BL-CTPP) or tobacco chitinase (Wt-TobChit-CTPP) do not bind any AtELP. Vacuolar targeting of both BL and TobChit are mediated by short stretches of hydrophobic residues that reside at the COOH-termini of the proteins (reviewed in Vitale and Raikhel, 1999). Unlike in the NTPP signals, it has not been possible to identify any common motif among the various CTPP signals identified thus far. Therefore, it has been suggested that a common secondary structure present within the CTPPs may serve as the sorting determinant that is recognized by a potential receptor, instead of a binding motif commonly found in the NTPPs. This potential receptor would most likely be involved in the transport of CTPP-containing proteins by a pathway different form that used by AtELP in the transport of NTPP-containing proteins. This possibility is supported by the differential sensitivity to wortmanin, of the two vacuolar sorting signals indicating the use of distinct sorting machinery by the two signals.

The involvement of AtELP in the NTPP-mediated pathway is further supported by results obtained from double immunogold labeling studies of ultra-thin root sections of transgenic Arabidopsis plants expressing either the vacuolar cargo reporter NTPP-Spo or BL-CTPP. These studies reveal the colocalization of AtELP with NTPP-Spo on the same Golgi cisternae, and in structures near the vacuole. However, AtELP is never observed to colocalize with BL-CTPP in similar experiments. The presence of multiple pathways to

the vacuole or lysosome has also been reported for mammalian and yeast systems. In yeast cells, Vps10p is involved in the sorting of a subset of vacuolar proteins, while others are transported by Vps10p-independent pathways (reviewed in Stack et al., 1995). In mammalian cells, there are M-6-PR-dependent and independent pathways of lysosomal targeting (Griffiths et al., 1988).

The results presented in this report demonstrate that the capability of AtELP to bind to the NTPP-sorting signals is linked to the *in vivo* targeting of proteins utilizing these signals. One of these proteins, NTPP-Spo, also colocalizes with AtELP in transgenic Arabidopsis plants. These results, taken together with data from our previous experiments involved in the characterization and localization of AtELP strongly support a role for AtELP as a sorting receptor in sorting through the NTPP-mediated mechanism in Arabidopsis cells.

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Chapter 5

Conclusions and Future Directions

The multifunctional vacuole is essential for plant cell survival and the proper maintenance of much of its function is dependent on the appropriate targeting of numerous proteins to the organelle. My thesis has involved the cloning and characterization of an Arabidopsis vacuolar sorting receptor, AtELP. The predicted amino acid sequence of AtELP indicates that it is a type I membrane protein that is structurally similar to many of the well-characterized mammalian and yeast protein sorting receptors and contains several conserved features, commonly found in those receptors (Chapter 2).

Antibodies raised against the recombinant *E. Coli* expressed AtELP were used as a tool to study the function of this protein in Arabidopsis, using several different approaches. Subcellular fractionation and immunogold labeling studies demonstrate that, AtELP is an integral membrane protein that is associated with several membrane fractions (Chapter 2 and 3). It is present in the *trans*-Golgi, a prevacuolar compartment (PVC) and a fraction enriched in clathrin and its associated adaptor containing vesicles. Moreover, the AtELP antibodies specifically recognized polypeptides in both monocots and dicots, indicating evolutionary conservation in plants (Chapter2). The potential role of AtELP in clathrin coated vesicle (CCV)-mediated intracellular protein transport from the TGN was analyzed by investigating its ability to interact with clathrin-associated adaptor proteins, in an *in vitro* binding assay. In this assay, the cytoplasmic tail of AtELP was found to interact specifically with the mammalian TGN-associated AP-1 adaptor proteins, but not with the plasma membrane-associated AP-2 adaptor proteins (Chapter 3).

A more direct involvement of AtELP in plant vacuolar targeting was analyzed by two different approaches. First, *in vitro* binding assays were used to show that AtELP interacts with the NH_2-terminal propeptide (NTPP) vacuolar sorting signals of barley aleurain (Aleu) and sweet potato sporamin (Spo), but not with the COOH-terminal propeptide (CTPP) targeting determinants of barley lectin (BL) and tobacco chitinase (TobChit). This interaction is both sequence-specific and pH-dependent. Second, electron microscopy studies in transgenic Arabidopsis plants, expressing either NTPP-Spo or BL-CTPP were used to show that the receptor colocalizes with the vacuolar cargo reporter protein, NTPP-Spo at the TGN and in structures near the vacuole, but not with BL-CTPP reporter protein (Chapter 4).

In the past, transport of soluble proteins through the plant secretory pathway to the vacuole has been studied in some detail at the level of sorting signals within the protein. My research has made significant contribution to the general progress towards understanding the molecular mechanisms of vacuolar protein sorting in plants. The isolation and functional analysis of AtELP, has provided substantial important information about the first step of the sorting mechanism, that is recognition of the sorting signals by a sorting receptor at the TGN. In addition, AtELP serves as an important marker that can enable us to differentiate between the various transport pathways to the different types of vacuoles. With the characterization of AtELP, that is likely to serve as a vacuolar sorting receptor, the route taken by proteins with NH_2-terminal sorting signals is becoming clear. Its preferential interaction with the NTPP-sorting signals and its co-localization with NTPP-Spo in Arabidopsis indicate that NTPP-containing proteins destined to the lytic vacuole (LV) are sorted by AtELP. The

receptor's localization at the *trans*-Golgi network (TGN), the PVC and in CCVs implicates these organelles in the transport of vacuolar proteins. Further, the biochemical characteristics of AtELP together with the preferential interaction of its cytoplasmic tail with 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.

Future work should be directed towards understanding the molecular mechanisms involved in the intracellular transport of vacuolar proteins by AtELP. In particular, the direct *in vivo* role of AtELP in sorting endogenous cargo proteins to the vacuole in Arabidopsis should be examined. Functional assays investigating the role of AtELP or its homologs in pea, BP-80 (Kirsch et al., 1994), and pumpkin, PV72/PV82 (Shimada et al., 1997), have been mostly restricted to *in vitro* binding experiments using synthetic peptides that correspond to vacuolar sorting signals of several proteins. Although, the use of this experimental approach was important for the original identification of a family of vacuolar protein sorting receptors from different plant species, it has also raised several new questions regarding the specificity of sequences recognized by this protein family. Based on these *in vitro* binding studies, there appears to be some variations in the type and sequences of the vacuolar sorting signals recognized by the receptor family, depending on the plant species. For example, BP-80 was originally identified and purified as a single protein from pea cotyledons based on its affinity for the NTPP-targeting determinants of barley aleurain and sweet potato sporamin (Kirsch et al., 1994). However, subsequent experiments demonstrated that it can also interact with the CTPP-based sorting signal present on Brazil nut 2S albumin (Kirsch et al., 1996). Although the

2S albumin signal contains a motif similar to the NPIR motif, found to be important for BP-80 binding to the aleurain and sporamin signals, this motif is not required for the receptor binding to the 2S albumin signal. Instead, a four-amino acid CTPP (IAGF) is required for binding but alone is not sufficient to permit interaction with BP-80. Subsequent molecular cloning of BP-80 from pea, revealed the presence of several other highly related cDNAs in this species. Thus, it is unclear as to which of these cDNAs encode for BP-80 that was originally identified from the pea CCV fraction, based on its binding properties. Similarly, the pumpkin homologs of AtELP/BP-80, PV72 and PV82, bind to both internal and CTPP regions of the pumpkin 2S albumin as well as the aleurain NTPP sorting signal *in vitro.* The internal propeptide domain contains a motif-NPWR-that is similar to that found in the aleurain and sporamin propeptides; however, this region is not critical for binding the peptide to PV72 and PV82 (Shimada et al., 1997). Instead, an NPLS-motif is essential for binding to the CTPP of the pumpkin 2S albumin. More recently, using a chemical cross-linking approach, a protein immunologically related to BP-80 was found to physically interact with a *Nicotiana alata* proteinase inhibitor precursor protein (Na-PI), that contains a C-terminal sorting signal, in stigma cells (Miller et al., 1999). Although this provided an *in vivo* evidence for the potential involvement of BP-80-related protein as a sorting receptor for this protein with a C-terminal sorting signal in stigma cells, however, there is no direct evidence from this experiment that the immunologically related protein from tobacco bound Na-PI because it specifically recognized the C-terminal propeptide of Na-PI. It is equally possible that a second, yet undetected sequence in Na-PI could have interacted with the potential BP-80 homolog in this experiment. The molecular details of this protein are not known at this

time. Results from the *in vitro* binding studies presented in my thesis demonstrate that the BP-80 related protein in Arabidopsis, AtELP, interacts specifically with the NTPPs of aleurain and sporamin, but not with the CTPPs of BL or TobChit. Moreover, the receptor colocalizes with NTPP-Spo and not CTPP-BL in transgenic Arabidopsis plants.

These results indicate the complex nature of protein sorting by this family of receptors in different plant tissues and species. Aleurain and sporamin are thought to travel from the TGN to the LV in CCVs, a process likely mediated by AtELP/BP-80. 2S albumins on the other hand, are storage proteins that have been shown to be transported by precursor-accumulating vesicles (PACs) to the protein storage vacuoles (PSVs). How is it that BP-80 isolated form pea CCVs can interact with sorting signals present on proteins destined for the PSVs? Conversely, how is it that PV72/82 isolated from PACs interact with the aleurain targeting signal?

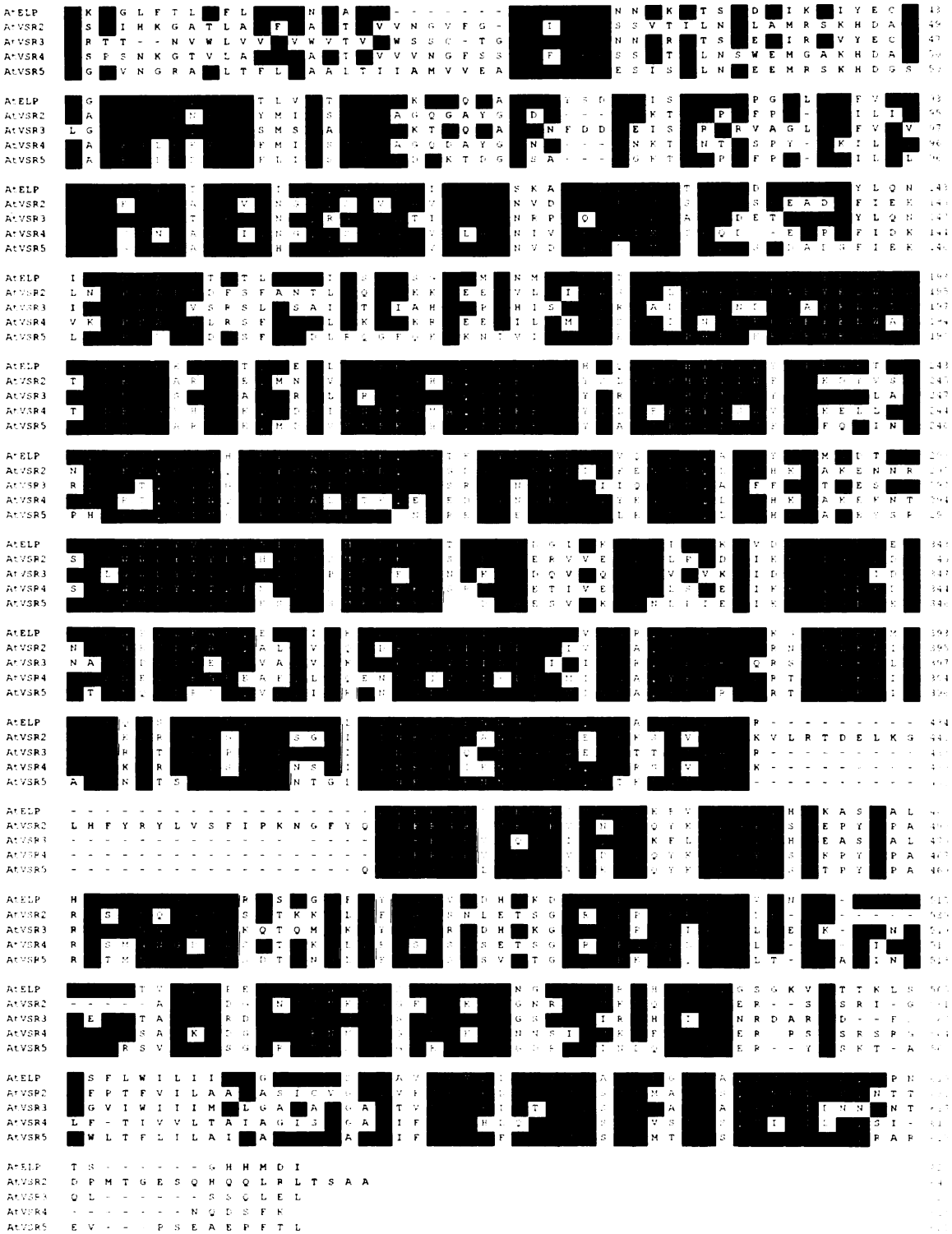
An outstanding feature of the AtELP/BP-80/PV72-82 family is that each species seems to have multiple members (Ahmed et al, 1997; Paris et al., 1997; Neuhaus and Rogers, 1998). Perhaps the complexity of the receptor binding can be explained by the existence of multiple isoforms that may be specific to certain tissues and substrates. These isoforms may function in the same pathway and each may either have developmental or tissue-specific expression, or different affinity for slightly different versions of the NTPP -targeting signals. Alternatively, the different AtELP family members may interact with the other vacuolar sorting signals identified in plants. Finally, members of the AtELP gene family may have redundant *in vivo* functions.

The evidence presented in my thesis all indicate a role for AtELP in the transport of NTPP-containing proteins in Arabidopsis. However the nature of its endogenous cargo

remains unknown, primarily because very few endogenous soluble vacuolar proteins have been characterized in this plant. This is due to the fact that many vacuolar proteins, or enzymatic activities associated with them, are found in both a vacuolar and secreted forms in Arabidopsis (Vitale and Raikhel, 1999). This dual localization has made it difficult to use them as endogenous reporters for studying vacuolar transport.

An understanding of the endogenous cargo sorted by AtELP becomes even more important, considering *AtELP* appears to be a member of a gene family. Several *AtELP*-related cDNAs or genomic clones from Arabidopsis have been identified in the Arabidopsis EST and genomic databases during the course of my project (Ahmed et al., 1997; Neuhaus and Rogers, 1998). The predicted amino acid sequence of the ORFs encoded by these nucleotide sequences show 60-80% identity to AtELP. An alignment of these sequences indicating sequence identity is presented in Figure 5.1, while a dendogram of the sequence identity is shown in Figure 5.2. We refer to the members of the gene family as AtVSR for *Arabidopsis thaliana* vacuolar sorting receptor, with AtELP being AtVSR1. Homology searches in the database showed two cDNA sequences having 81 and 63% homology with *AtELP*. These sequences are *AtVSR2* (accession # U79960) and *AtVSR3* (accession # 110G6T7). The complete genomic sequences of *AtELP* (accession # AL132969.1), *AtVSR2* (accession # ATAC004705, pid 3252815) and *AtVSR3* (accession # AL021637, pid g 2827665) together with two other clones presenting homologies to *AtELP* have been deduced from the analysis of the Arabidopsis genomic database. These clones have been named *AtVSR4* (accession # ATAC002338, pid g 2347209) and *AtVSR5* (accession # ATAC004238, pid g 3033390) and their nucleic acid showed 84 and 61% homology with *AtELP*, respectively. *AtELP* is located on

Figure 5.1
Alignment of the predicted amino acid sequences encoded by the ORFs of AtELP and several related cDNAs and genomic clones. The highlighted areas indicate sequence identity. AtVSR (Arabidopsis thaliana vacuolar sorting receptor).


AtELP
AtVSR2
AtVSR3
AtVSR4
AtVSR5

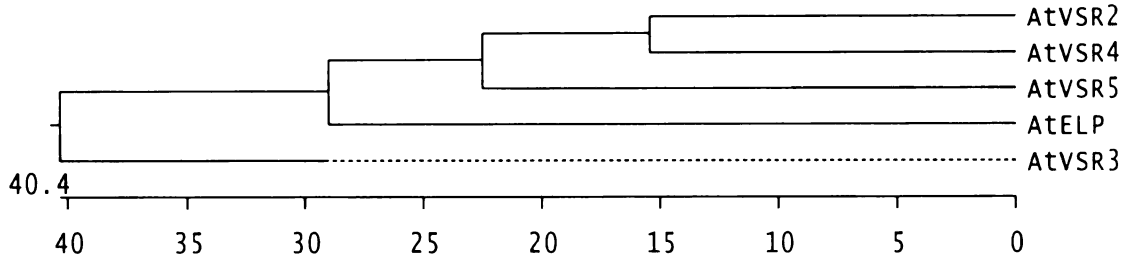


Figure 5.2
Phylogenetic tree of the predicted amino acid sequences encoded by the ORFs of AtELP and several related cDNAs and genomic clones. AtVSR (Arabidopsis thaliana vacuolar sorting receptor). Numbers below indicate approximate deviation from potential ancestral protein.

chromosome III, *AtVSR2*, *4* and *5* are all on chromosome II, while *AtVSR3* is located on chromosome IV. The gene family is composed of at least five genes having 11-13 exons and 10-12 introns.

As a first step towards understanding the specific function of this gene family members in Arabidopsis, the tissue and developmental stage-specific expression of each gene could be analyzed at both the RNA and protein level. Gene-specific probes could be synthesized either by subcloning nucleotide sequences that are specific to each or by PCR techniques. Alternatively, RT-PCR techniques could be used with oligonucleotide primers specific to each gene, to reverse transcribe potential cDNA products from the expressed RNAs corresponding to the genes. If no differential expression of the five members of this gene family is found, it is likely that they have either a redundant function in vacuolar targeting, or that each is involved in sorting specific vacuolar cargo proteins in the same cell type. To address this question, several approaches could be taken. The first could involve isolating knock out mutants for each gene, containing either T- DNA or transposable element insertions. This reverse genetics approach has been successfully used by several other groups to understand the function of different members of a gene family (Mckinney et al., 1995, Hirsch et al., 1998) in plants. These knock out mutants could then be used to study the function of each gene by following the fate of endogenous vacuolar reporters or those in transgenic Arabidopsis lines overexpressing either NTPP-Spo or BL-CTPP, by crossing the mutant lines to the transgenic lines. The targeting of vacuolar proteins in the mutants could be analyzed either by immunogold labeling, followed by visualization using electron microscopy, or by pulse chase analysis. These studies could lead to an understanding of any cargo

selective function of each members of the protein family. Alternatively, an antisense or dominant negative approach could be utilized, by overexpressing the ORFs from each member of the gene family, under a constitutive promoter, in an antisense orientation or as a dominant negative mutant containing only the ligand binding lumenal portion in transgenic plants. However, it is important to notice that given the high degree of sequence identity between all the members of this gene family, a complete knock out of all the receptors could be detrimental to the plant.

An additional approach could involve an examination of a direct physical interaction between AtELP and a vacuolar protein by chemical cross-linking. Unfortunately, there are no well-characterized endogenous vacuolar proteins in Arabidopsis. Thus, we will have to rely on using transgenic Arabidopsis plants expressing the vacuolar reporter protein, NTPP-Spo, which appears to be sorted by AtELP. To investigate a direct physical *in vivo* interaction between, AtELP and NTPP-Spo, AtELP-enriched subcellular fractions could be prepared from the NTPP-Spo plants and exposed to membrane permeable chemical cross-linkers that are either thiol cleavable (such as, DSP) or non-cleavable (such as, DSG or DSS), before vesicles are lysed by detergent. AtELP- or NTPP-Spo-specific complexes could then be immunoprecipitated using antibodies to AtELP or NTPP-Spo and preimmune sera as controls. The immunoprecipitated complexes could then be analyzed by SDS-PAGE in the presence or absence of reducing agents followed by immunoblotting with anti-AtELP and anti-NTPP-Spo antibodies. If AtELP and NTPP-Spo physically interact with each other *in vivo*, the immunoprecipitated interacting complex should contain both the proteins. Their presence should be detectable in subsequent Western blot analyses. In addition, these cross-linking

studies could be carried out in wild type cells, to identify the endogenous AtELP-interacting proteins, some of which may be its cargo ligand(s). Again, the presence of several putative AtELP-related proteins may complicate these experiments. Therefore, AtELP-specific antibodies have to be used during these immunoprecipitation studies, which may be difficult to generate given, the high degree of similarity between the predicted amino acid sequences of each of the AtELP-related ORFs identified thus far. In that case, peptide-specific antibodies could be used to detect each protein in the family. In addition to being useful in detecting the tissue or stage-specific expression of each protein, these antibodies could be used in biochemical fractionation and immunogold labeling studies to investigate their subcellular distribution and localization, respectively. The antibodies could be raised against peptides specific to each protein's large lumenal domains. The lumenal-specific antibodies could be useful for both western analysis and electron microscopy, as both of these approaches use detergent, which should extract these proteins from the membranes, thus exposing their lumenal portions, that are otherwise inside the vesicles. These peptide-specific antibodies could shed some light on whether each member is present in different membrane compartments, and thus involved in targeting through different pathways.

Finally, each protein could be tagged with an epitope (T7, HA, etc) at either the NH_2-terminus or COOH-terminus to differentiate them from each other. Subsequently, a detailed analysis of their subcellular distribution and localization could be carried out in the transgenic Arabidopsis lines overexpressing the epitope-tagged versions of each receptor. Further, these tagged lines could be used to isolate receptor-cargo interacting complexes that are specific to each type of protein, revealing any cargo specificity.

Moreover, COOH-terminal epitope tagged AtELP or its related proteins could be used to purify the corresponding transport vesicles to identify potential cargo proteins that might be sorted specifically by one member of the family and the others. This approach has been successfully used by a number of different research groups to investigate similar questions (Zheng et al., 1999; Wandinger-Ness et al.,1990).

Based on the results presented in this thesis, a working model for the role of AtELP can be developed from these observations, as well as studies from other systems. A schematic representation of this model is shown in Figure 5.3. In this model, the lumenal portion of AtELP binds to NTPP-containing proteins under neutral pH conditions at the TGN, where soluble vacuolar proteins are sorted away from those that are destined for secretion. The cytoplasmic tail of AtELP on the other hand, interacts with the TGN-associated AP-1 adaptor protein complex to initiate the formation of CCVs and package the receptor-ligand complex into these vesicles. These vesicles then travels to a PVC where, under the acidic pH conditions of this compartment, AtELP dissociates from its ligand. The ligand, is further transported to the vacuole, while AtELP is be recycled back to the TGN for further rounds of sorting.

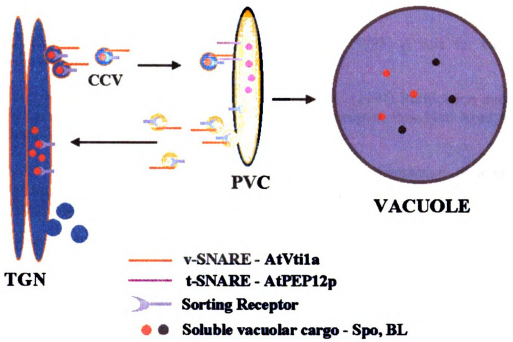


Figure 5.3
A working model for transport of vacuolar proteins by AtELP. The sorting receptor, AtELP, would interact with its ligand, a soluble vacuolar cargo protein (NTPP-sporamin) at the TGN. This receptor-ligand complex would then be packaged into AP-1 containing clathrin coated vesicles (CCVs), which bud off from the TGN. In addition, these vesicles contain the v-SNARE, AtVTI1a. These transport vesicles then travel a prevacuolar compartment (PVC), where AtVTI1a interacts with the t-SNARE, AtPEP12p, initiating the vesicle docking and fusion steps. At PVC, AtELP would release its cargo (see text for details) and the receptor would be recycled back to the TGN for further rounds of sorting. The cargo protein is further transported to the vacuole by an unknown mechanism.

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