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THE ROLE OF THE *ARABIDOPSIS* PHOSPHATIDYLINOSITOL 3-KINASE IN VACUOLAR PROTEIN TRANSPORT

by

Christopher A. Jones

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Biology

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2002

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ABSTRACT

The Role of the *Arabidopsis* Phosphatidylinositol 3-kinase

in Vacuolar Protein Transport

by

Christopher A. Jones, Master of Science

Utah State University, 2002

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Department: Biology

In all eukaryotic organisms vesicle-mediated protein transport is crucial for maintaining cellular homeostasis. Over the past decade significant strides have been made in elucidating the molecular mechanisms governing this complex process. Both genetic and biochemical studies have demonstrated the unequivocal involvement of class III phospatidylinositol 3-kinases (PtdIns 3-kinases) in regulating vesicle transport. While PtdIns 3-kinases have been well studied in both yeast and mammalian systems, the role of these enzymes in plants is poorly understood. In the present study, we demonstrate that the *Arabidopsis* PtdIns 3-kinase is localized to the cytoplasm, and perhaps the Golgi apparatus. Moreover, we show that reduction in cellular PtdIns(3)P levels is correlated with missorting of a vacuolar marker protein. Altogether, our data suggest that AtVps34p (and its lipid product PtdIns(3)P) is involved in regulating vesicle-mediated protein transport to the vacuole in *Arabidopsis*.

(59 pages)

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ABBREVIATIONS

AP-1	Adaptor Protein
At	Arabidopsis thaliana
AtELP	At Epidermal Growth Factor Receptor-like Protein
BiP	Binding Protein
BP-80	Binding Protein
CTPP	Carboxy Terminal Pro-Peptide
DAG	Diacylglycerol
EEA1	Early Endosomal Autoantigen 1
EGFP	Enhanced Green Fluorescent Protein
ER	Endoplasmic Reticulum
ERD2	ER Retention Defective
FAB	Formation of Aploid and Binucleate
FYVE	Fab1 YGL023 Vps27 EEA1
HDEL	Histidine Aspartic Acid Glutamic Acid Leucine
HRS	Hepatocyte Growth Factor Regulated Tyrosine Kinase Substrate
IP_3	Inositol Trisphosphate
KDEL	Lysine Aspartic Acid Glutamic Acid Leucine
NBD	7-nitrobenz-2-oxa-1,3-diazole
NSF	N-ethylmaleimide Sensitive Factor
NTPP	Amino Terminal Pro-Peptide
PH	Pleckstrin Homology
PLC	Phospholipase C
PtdIns	Phosphatidylinositol
PtdIns(3)P	Phosphatidylinositol 3-phosphate
PtdIns(4)P	Phosphatidylinositol 4-phosphate
PtdIns(3,4)P_2	Phosphatidylinositol 3,4-bisphosphate
PtdIns(4,5)P_2	Phosphatidylinositol 4,5-bisphosphate
PtdIns(3,4,5)P_3	Phosphatidylinositol 3,4,5-trisphosphate
SNAP	Soluble NSF Attachment Protein
SNARE	SNAP Receptor
TGN	*trans*-Golgi Network
VAC	Vacuole Segregation Mutants
VPS	Vacuolar Protein Sorting Mutants

CHAPTER I

INTRODUCTION

The Plant Cell Secretory Pathway

Transport from the Endoplasmic Reticulum to the Golgi Complex

Plant secretory proteins destined for the vacuole are typically synthesized on ribosomes associated with the endoplasmic reticulum (ER). The nascent polypeptide chain emerging from the ribosome is cotranslationally inserted into the lumen of the ER via an N-terminal signal peptide, which is subsequently cleaved by a signal peptidase on the lumenal side of the ER membrane (Vitale et al., 1993). In the oxidizing environment of the ER lumen, translocated polypeptides attain their appropriate tertiary conformation through interaction with the ER-resident protein BiP and receive post-translational modifications. Proteins that are identified as incompletely or incorrectly folded are retained and degraded in the ER by a mechanism that does not require movement to the lytic vacuole (Pedrazzini et al., 1997). Alternatively, misfolded proteins can be returned to the cytosol by movement through the translocation machinery (Pedrazzini et al., 1997; Frigerio et al., 1998) and degraded by the ubiquitin-proteasome pathway.

Secretory proteins contain sorting information in the form of a stretch of conserved amino acid residues in a defined location within the mature polypeptide, that is ultimately responsible for conferring localization to a particular compartment along the secretory pathway. For example, proteins with the tetrapeptide HDEL, KDEL or a variant of these sequences are targeted to and retained in the lumen of the ER (Herman et al., 1990; Denecke et al., 1992). If an HDEL-containing protein is inappropriately

delivered to the Golgi complex, it is efficiently retrieved through a system that recognizes the ER-retention moiety (Pelham, 1988). Recently, Boevink and colleagues (1998) demonstrated that this retrograde (Golgi to ER) pathway exists in plants, by using a green fluorescent protein/*Arabidopsis* ERD2 (ER-retention defective) receptor fusion protein. Secretory proteins in the lumen of the ER that lack an HDEL-like sequence await export via anterograde (ER to Golgi) transport vesicles. Presently, two models describing this process have been advanced, active transport and bulk flow. The former implies involvement of a sorting receptor that recognizes and binds to cargo protein in the lumen of the ER and facilitates vesicle budding. The latter suggests passive diffusion of cargo protein into spontaneously budding vesicles. While recent data seems to support the active sorting model (Barlowe et al., 1994; Balch et al., 1994), efforts to identify an ER export receptor and an ER exit signal on vacuolar and secreted proteins have been unsuccessful (Vitale and Denecke, 1999). Interestingly, several reports suggest the existence of multiple export pathways from the ER (Gomez and Chrispeels, 1993; Jiang and Rogers, 1998), each characterized by its relative sensitivity to Brefeldin A. More specifically, Hara-Nishimura and colleagues (1998) have postulated that non-glycosylated storage proteins contained within 200-400 nm diameter electron dense vesicles bypass the Golgi complex, but are nonetheless delivered to the protein storage vacuole. Conversely, glycosylated storage proteins are delivered to the Golgi complex and eventually sorted to the protein storage vacuole through events at the *trans*-Golgi network (TGN) (Hara-Nishimura et al., 1998).

Intra-Golgi Transport

Secretory proteins that are not retained in the ER nor delivered directly to the protein storage vacuole are transported to the Golgi complex in phospholipid vesicles for further posttranslational modification (Figure 1). The mechanism whereby vesicles bud from and fuse to particular intracellular membranes is exquisitely complex. A number of the molecular components (including Rab GTPases, SNAREs and Sec1p homologs) that preserve the fidelity of this process have been identified in plants (reviewed in Sanderfoot and Raikhel, 1999), however, the intracellular distribution of many of these proteins is not conserved between plants and other eukaryotes, such as the budding yeast *Saccharomyces cerevisiae*.

In plant cells, the Golgi complex consists of hundreds of individual "units," each of which is defined as a Golgi stack and a TGN (Marty, 1978; Staehelin and Moore, 1995; Dupree and Sherrier, 1998). Each Golgi stack consists of three spatially and biochemically discrete cisternae: *cis*, *medial*, and *trans*. While it is currently postulated that secretory proteins are transported between the individual Golgi subcompartments via a "maturation" mechanism, in which earlier cisternae mature into later cisternae (i.e., *cis* → *medial* → *trans*) and vesicles originating from the ER regenerate the *cis*-cisternae (Glick et al., 1997; Pelham, 1998), additional evidence suggests that either vesicular transport (Orci et al., 1997) or vesiculotubular structures (Orci et al., 1998) may be involved in this process. In addition to anterograde traffic through the Golgi stack, it is hypothesized that retrograde transport of cisternal-specific proteins occurs as well, although this mechanism in plant cells is not currently understood (Bednarek and Raikhel, 1992).

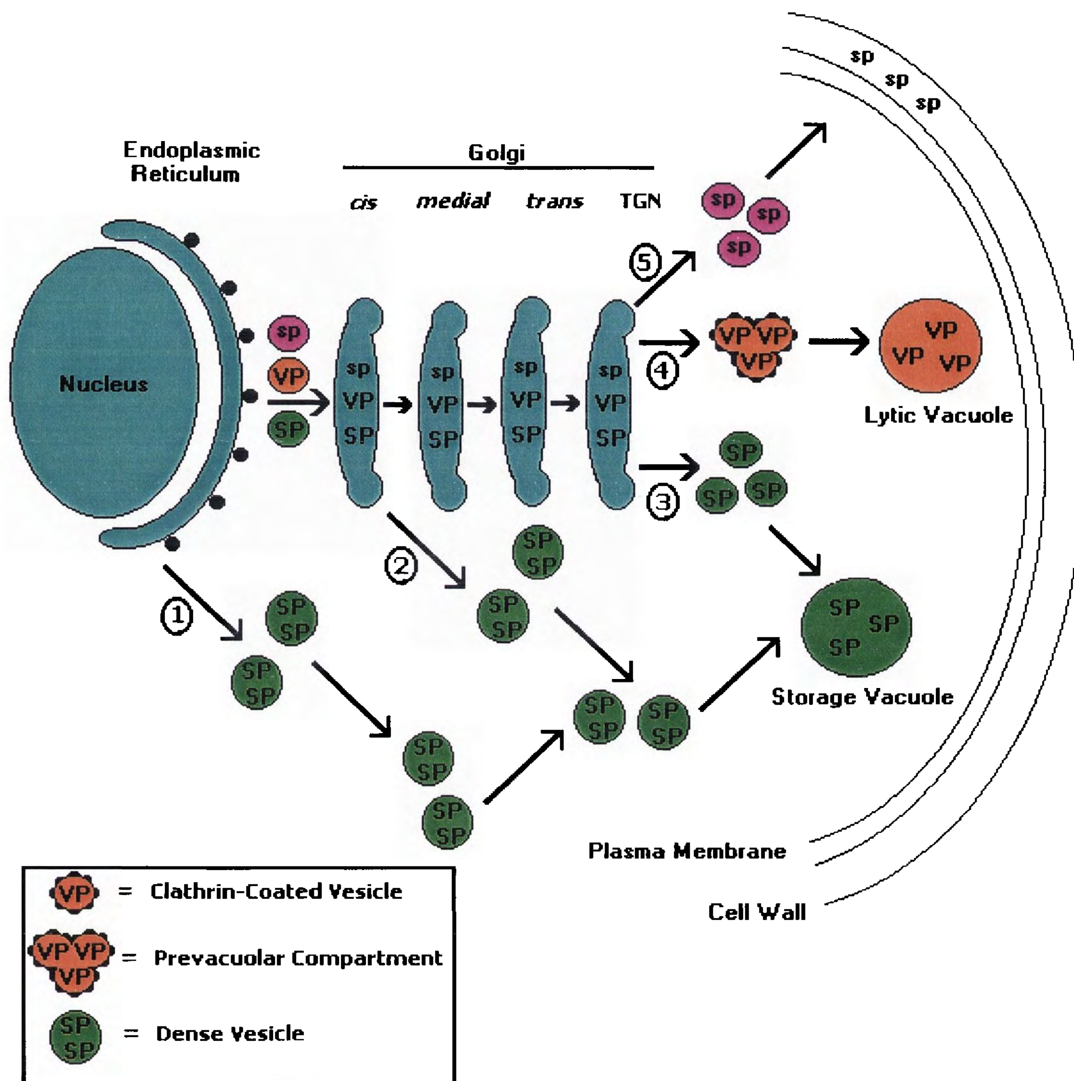

Figure 1. A Model Depicting Protein Movement Through the Plant Cell Secretory Pathway.

Specific classes of plant cell secretory proteins are delivered to different cellular locations. Storage proteins can be delivered to the storage vacuole from the endoplasmic reticulum (1); the *cis*-Golgi (2); or the *trans*-Golgi Network (TGN) (3) in dense vesicles. Vacuolar proteins are transported to the lytic vacuole in clathrin-coated vesicles through a prevacuolar compartment (4). Secreted proteins are transported to the plasma membrane for egress from the cell (5). sp, secreted protein; SP, storage protein; TGN, *trans*-Golgi network; VP, vacuolar protein.

trans-Golgi Network-to-Vacuole Transport

Secretory proteins that reach the *trans*-Golgi network are either transported to the vacuole or delivered to the cell surface for exocytosis (Figure 1). Recent evidence has indicated the presence of two functionally distinct types of vacuoles in plant cells, as well as multiple biochemical pathways for delivery of protein cargo to these organelles (Paris et al., 1996; Swanson et al., 1998). One type of vacuole is utilized by the plant cell as a storage site for particular proteins, and is hence called a protein storage vacuole. The second type of vacuole is a highly acidic, degradative organelle, which is comparable in function to the animal cell lysosome and yeast cell vacuole, and acts principally in protein turnover. It has been hypothesized that fusion of these two distinct vacuoles occurs in mature plant cells as a mechanism by which carbon and nitrogen can be mobilized for specific intracellular processes (Paris et al., 1996). Both vacuolar types have been demonstrated to exist within a single cell, and receive their component protein cargo through the secretory pathway.

In light of data indicating the presence of multiple biochemically unique vacuoles, it is not surprising that Hohl and colleagues (1996) have suggested that transport of proteins from the Golgi to the lytic and protein storage vacuole occur via distinct phospholipid vesicle populations. In particular, storage proteins are transported to the protein storage vacuole in electron dense vesicles while soluble proteins destined for the lytic vacuole exit the *trans*-Golgi network in clathrin-coated vesicles. It is interesting, moreover, that clathrin-coated vesicles have been observed budding from dense vesicles that have exited the *trans*-Golgi network, suggesting a possible salvage mechanism for proteins (hydrolytic enzymes in particular) mispackaged in the Golgi (Hinz et al., 1993;

Hohl et al., 1996). While the preponderance of available data suggests that the *trans*-Golgi network is the principal branch point in the secretory pathway, a recent report has indicated that vacuolar storage proteins in developing pea cotyledons may be sorted in the *cis*-cisternae of the Golgi stack (Hillmer et al., 2001), adding further complexity to the protein secretory pathway in plant cells.

Vacuolar Sorting Signals and Receptors

Proteins destined for either the lytic vacuole or protein storage vacuole must contain one of three types of sorting determinants (Chrispeels and Raikhel, 1992). Sweet potato sporamin (Nakamura et al., 1993) and the barley cysteine protease aleurain (Holwerda et al., 1992) contain an N-terminal propeptide (NTPP). Within the NTPP is a conserved amino acid sequence, Asn-Pro-Ile-Arg, which is necessary for transit of these proteins to the vacuole (Nakamura et al., 1993; Matsuoka et al., 1995; Paris et al., 1997). The mechanism whereby NTPP-containing proteins are identified and sequestered in TGN-derived vesicles is analogous to carboxypeptidase Y sorting in yeast (Marcusson et al., 1994) and carbohydrate-mediated protein sorting in mammalian cells (Kornfeld and Mellman, 1989; Kornfeld, 1992). Specifically, the Asn-Pro-Ile-Arg motif in these proteins interacts with the lumenal domain of a vacuolar sorting receptor. In plants, two such receptors have been identified, BP-80 from pea cotyledons (Kirsch et al., 1994), and AtELP from *Arabidopsis* (Ahmed et al., 1997) (Figure 2). Each of these single-pass transmembrane proteins contains a lumenal domain with cysteine-rich repeats, and a short cytoplasmic domain for interaction with the AP-1 adaptor complex (Sanderfoot and Raikhel, 1999). Moreover, each of these receptor proteins has been located in the *trans*-

cisternae and in the prevacuolar compartment through immunogold electron microscopic analysis (Paris et al., 1997; Sanderfoot et al., 1998).

Other proteins, such as barley lectin (Bednarek and Raikhel, 1992; Matsuoka et al., 1995) and tobacco chitinase (Neuhaus et al., 1994), contain a C-terminal

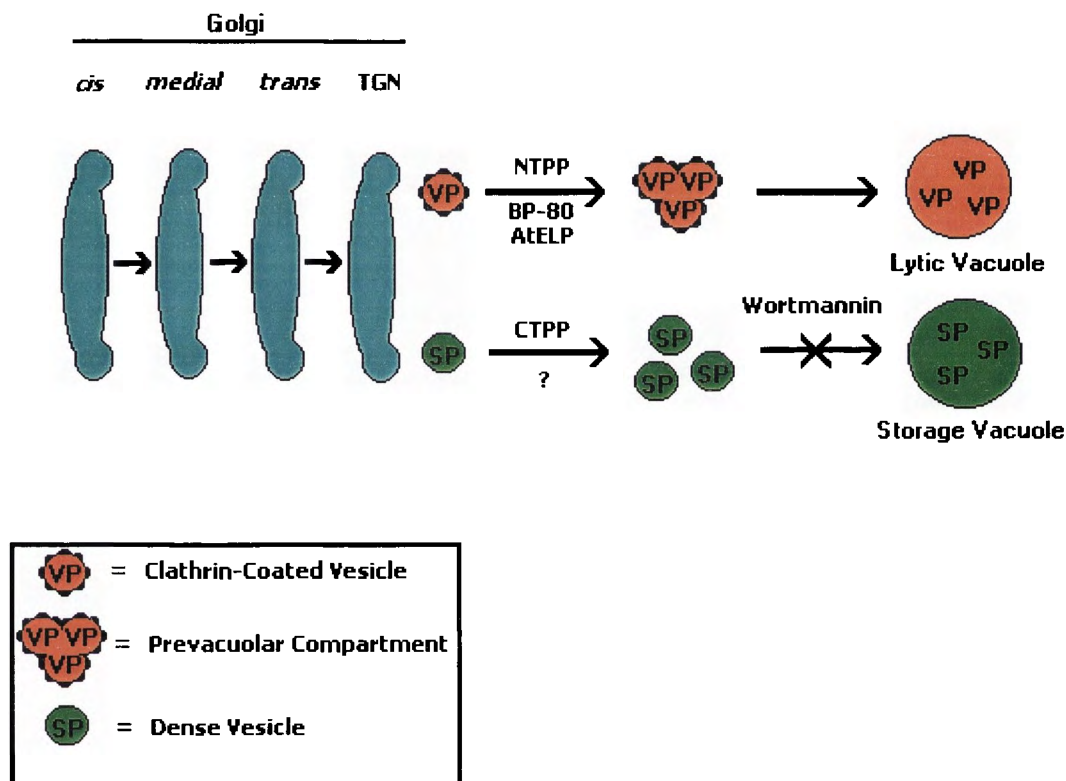

Figure 2. The Two-Pathway Paradigm Describing *trans*-Golgi Network-to-Vacuole Protein Transport in Plant Cells.

Vacuolar proteins with an NTPP interact with the lumenal domain of a sorting receptor (i.e., BP-80 or AtELP), are packaged into clathrin-coated vesicles and transported to the lytic vacuole through an intermediate (or prevacuolar) compartment. Conversely, vacuolar proteins with a CTPP are packaged into dense vesicles and delivered to the storage vacuole in a pathway that is sensitive to wortmannin. It is currently unclear whether CTPP-containing proteins interact with a sorting receptor, although this is a likely scenario. CTPP, C-terminal propeptide; NTPP, N-terminal propeptide; SP, storage protein; VP, vacuolar protein.

propeptide (CTPP). While a consensus vacuolar targeting sequence has not been identified within the C-terminal domain, mutational analysis has revealed that hydrophobic residues are important for targeting barley lectin to the vacuole (Dombrowski et al., 1993). Presently, a vacuolar sorting receptor that interacts specifically with CTPP-containing proteins has not been identified. However, a recent report has demonstrated that BP-80 complexes with a CTPP-containing ~46-kDa proteinase inhibitor precursor protein (Na-PI) from *Nicotiana alata* (Miller et al., 1999). Furthermore, it was demonstrated that removal of the C-terminal domain of Na-PI prevented deposition of this protein in the vacuole (Miller et al., 1999). These data are not altogether surprising since several reports have indicated that *in vitro*, BP-80 is capable of interacting with the C-terminal motif of some albumins (Kirsch et al., 1996; Shimada et al., 1997). Interestingly, the CTPPs of both barley lectin and the *Arabidopsis* 2S albumin are unable to complex with BP-80 (Kirsch et al., 1996). Whether BP-80 and/or its orthologs are capable of interacting with storage proteins *in vivo* and transporting them to the protein storage vacuole remains unclear.

While the data presented by Miller and colleagues (1999) is exciting, several conclusions from their study have been questioned (Jiang and Rogers, 1999; Miller and Anderson, 1999), primarily because they contradict the "two-pathway" paradigm describing vesicular traffic to the vacuole in plants (Figure 2). Briefly, one such pathway is defined by the presence of the vacuolar sorting receptor BP-80 and involves protein transport from the Golgi complex to the prevacuolar compartment (serving the lytic vacuole) in clathrin-coated vesicles. The other pathway is characterized by the transport of storage proteins to the protein storage vacuole in dense vesicles. In an elegant study,

Hinz and colleagues (1999) demonstrated that clathrin-coated vesicles exiting the Golgi are significantly enriched in BP-80, while dense vesicles are devoid of this vacuolar sorting receptor. Moreover, dense vesicles contain abundant seed-type storage protein, whereas clathrin-coated vesicles do not. These data seemingly suggest that proteins containing an N-terminal sorting determinant are transported in the BP-80/clathrin-coated vesicle pathway, while proteins with a C-terminal sorting determinant are transported in the dense vesicle pathway (Figure 2). But, Miller et al. (1999) have identified a CTTP-containing protein that interacts with BP-80 and is delivered to a proteolytically active vacuole. At issue with Jiang and Rogers (1999) is whether the CTPP is truly responsible for directing Na-PI to the lytic vacuole, or whether another sorting signal (presently unidentified) is involved. A more detailed biochemical analysis of the interaction between Na-PI and BP-80 will be necessary to distinguish between these possibilities.

Additional experimental evidence for the "two pathway" paradigm has emerged from a study using the lipid kinase inhibitor wortmannin. Specifically, delivery of a CTPP-containing protein to the vacuole was compromised in the presence of the inhibitor, whereas an NTPP-containing protein was accurately transported (Matsuoka et al., 1995) (Figure 2). While several NTPP sorting receptors have been identified, a CTPP sorting receptor has not.

Finally, proteins that do not have a cleavable propeptide, such as phytohemagglutinin (Tague et al., 1990) and legumin (Saalbach et al., 1991) contain targeting determinants within the mature protein. A sorting receptor for this type of vacuolar protein has not been identified and the mechanism whereby such proteins are delivered to the vacuole is not presently understood.

Membrane Transport in Yeast and Mammalian Cells

Over the past decade, genetic studies in the budding yeast *Saccharomyces cerevisiae* have contributed significantly to our understanding of membrane trafficking dynamics in eukaryotic cells. Of particular interest has been elucidation of the mechanism regulating vesicle-mediated protein traffic from the Golgi complex to the vacuole, an acidic organelle that contains hydrolytic enzymes and is functionally comparable to the mammalian cell lysozome. The yeast secretory pathway is generally analogous to that of plants.

Genetic screens developed by Emr, Stevens and colleagues have facilitated the identification of more than 40 *V*acuolar *P*rotein *S*orting (*VPS*) genes that are necessary for delivery of soluble proteins to the yeast vacuole (Odorizzi et al., 2000). Characterization of the *VPS* genes led to identification of *VPS34*, a gene encoding a PtdIns 3-kinase that phosphorylates PtdIns on the D-3 position of the inositol ring to yield PtdIns(3)P (Schu et al., 1993) (Figure 3). Specific mutations in the phospholipid kinase domain of *VPS34* result in both reduced cellular levels of PtdIns(3)P and defective vacuolar protein transport (Schu et al., 1993). Additionally, when mutant cells with a temperature-conditional allele of *VPS34* are shifted to a non-permissive temperature, they rapidly missort and secrete the vacuolar hydrolase carboxypeptidase Y (Stack et al., 1995a), suggesting that the anterograde flow of proteins from the Golgi complex to the vacuole is regulated by the PtdIns 3-kinase encoded by *VPS34*.

Further characterization of the *VPS* genes led to identification of Vps15p, a

membrane-associated serine/threonine protein kinase (Herman et al., 1991a,b). Biochemical studies have revealed that Vps15p exists in a complex with Vps34p *in vivo* (Stack et al., 1993). Specifically, Vps15p recruits Vps34p from the cytosol to an intracellular membrane, arguably a late Golgi/endosomal compartment (Herman et al., 1991b; Stack et al., 1993; Stack et al., 1995b). Membrane recruitment is dependent on Vps15p activity, and formation of the Vps15p-Vps34p heterodimer significantly increases PtdIns3-kinase activity (Stack et al., 1993). These data suggest that Vps15p regulates both Vps34p localization and activity, and by logical extension, vacuolar protein transport. Compellingly, mutants with a carboxy-terminal deletion of *VPS15* display a temperature-sensitive defect in PtdIns(3)P levels and in delivery of acid hydrolases to the vacuole (Herman et al., 1991a; Stack et al., 1995b). Altogether, these results imply a fundamental role for PtdIns(3)P in vesicle-mediated transport from the Golgi complex to the vacuole in yeast.

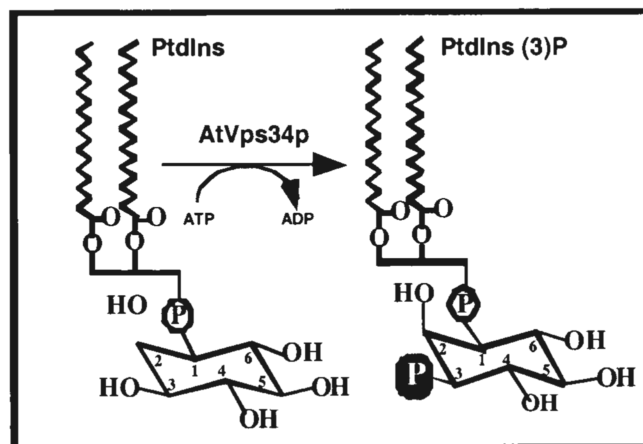

Figure 3. Class III PI 3-kinases Synthesize PtdIns(3)P.

Class III PI 3-kinases (of which AtVps34p is a member) phosphorylate the D-3 position of the inositol ring of PtdIns to produce PtdIns(3)P.

The human homologs of *VPS34* and *VPS15* have been cloned (Volinia et al., 1995; Panaretou et al., 1997). These genes encode a PtdIns 3-kinase (hVps34p) and a myristilated serine/threonine protein kinase (p150), respectively. As in *S. cerevisiae*, p150 physically interacts with hVps34p *in vivo* and enhances its lipid kinase activity (Panaretou et al., 1997). Moreover, studies using the PI 3-kinase inhibitor wortmannin have demonstrated the requirement for PtdIns(3)P in mammalian protein transport. Specifically, nanomolar concentrations of the fungal metabolite wortmannin (which inhibit PtdIns 3-kinase activity) also impedes processing and delivery of cathepsin D to the lysozome (Brown et al., 1995). Similarly, delivery of platelet-derived growth factor receptors from the plasma membrane to the lysozome was disrupted in cells either treated with wortmannin or injected with anti-hVps34p antibodies (Shpetner et al., 1996; Siddhanta et al., 1998). In concert, these data indicate that membrane transport to the hydrolytic compartment in both yeast and mammalian cells is functionally regulated by the Vp34p-Vps15p complex, and its lipid product, PtdIns(3)P.

Phosphoinositide Metabolism

Phosphoinositides (PIs) are a class of membrane phospholipids comprised of phosphatidylinositol (PtdIns) and its phosphorylated derivatives. The inositol head group of PtdIns can be reversibly phosphorylated (by lipid kinases) at multiple positions (3', 4' or 5') to generate a variety of unique, membrane-bound molecules. Until only recently, phosphoinositdes were believed to be static structural components of phospholipid bilayers. Berridge (1993) originally demonstrated that phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P_2) can be hydrolyzed by phospholipase C (PLC) to produce

soluble inositol (1,4,5)-trisphosphate (IP_3) and membrane-bound 1,2-diacylglycerol (DAG), which subsequently stimulate calcium mobilization and protein kinase C activation, respectively. This novel observation implied that phosphoinositides might act as classical second messenger molecules. It is presently appreciated that phosphoinositides function as important regulatory elements in a variety of complex eukaryotic signaling pathways, including cytoskeletal rearrangement, membrane transport and differentiation (reviewed in Martin, 1998).

While certain phosphoinositide species are utilized as substrates for hydrolytic enzymes (as detailed for PtdIns(4,5)P_2) others function independently as second messengers. In particular, the products of phosphoinositide 3-kinases (PI 3-kinases) do not appear to be substrates for intracellular phospholipases (Serunian et al., 1989). Phosphatidylinositol (3)-phosphate (PtdIns(3)P) is bound by effector proteins such as the early endosomal autoantigen 1 (EEA1) during membrane transport (Stenmark et al., 1996; Patki et al., 1997). Phosphatidylinositol (3,4)-bisphosphate (PtdIns(3,4)P_2) and phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P_3) can be bound by the pleckstrin homology (PH) domain of 3-phosphoinositide-dependent kinase-1 (PDK1), which in turn activates the AKT/PKB signaling pathway (reviewed in Alessi and Downes, 1998). Moreover, PtdIns(3,4,5)P_3 is capable of directly activating PLC during calcium-dependent processes (Scharenberg and Kinet, 1998).

Interestingly, the aforementioned lipid products (PtdIns(3)P, PtdIns(3,4)P_2, PtdIns(3,4,5)P_3) are each synthesized by unique classes of PI 3-kinases, which likely utilize specific substrates *in vivo*. Specifically, the class I enzymes, which consist of the

p110/p85 heterodimer and the G-protein linked p110γ use PtdIns(4)P and PtdIns(4,5)P_2 as substrates (Kapeller and Cantley, 1994; Stoyanov et al., 1995; Stephens et al., 1997).

The class II enzymes are 170-210 kDa polypeptides, contain a characteristic COOH- terminal C2 homology domain and use PtdIns and PtdIns(4)P as substrates (MacDougall et al., 1995; Virbasius et al., 1996; Domin et al., 1997). The class III enzymes consist of the *S. cerevisiae* Vps34p and its homologs in other species (Herman and Emr, 1990; Welters et al., 1994; Volinia et al., 1995; Takegawa et al., 1995; Zhou et al., 1995; Linassier et al., 1997). These enzymes utilize only PtdIns as a substrate and for this reason are referred to as PtdIns-specific 3-kinases, or PtdIns 3-kinases.

Plant Phosphatidylinositol 3-kinases

PtdIns 3-kinase homologues have been identified in both soybean (Hong and Verma, 1994) and *Arabidopsis* (Welters et al., 1994). The deduced amino acid sequences of these cDNA clones strongly resemble the class III PtdIns 3-kinase. In particular, AtVps34p shares approximately 40% amino acid sequence identity with the aforementioned yeast Vps34p (Welters et al., 1994), and has been demonstrated to synthesize PtdIns(3)P upon inducible expression in *E.coli* (DeWald, unpublished observations).

The cellular function of plant PtdIns 3-kinases has not been clearly defined, and seems to vary from one species to the next. Originally, Hong and Verma (1994) demonstrated that PtdIns 3-kinase activity was critical for development of the peribacteroid membrane in soybean. Then, using anti-sense mRNA constructs, Welters and colleagues (1994) postulated that the *Arabidopsis* Vps34p was crucial for general

plant growth and development. More recently, a study using the lipid kinase inhibitor wortmannin suggested a role for PtdIns 3-kinase in vacuolar protein trafficking in tobacco BY-2 suspension cells (Matsuoka et al., 1995).

Presently, two reports have emerged which add further complexity to the role of class III PtdIns 3-kinases in plants. Unexpectedly, Bunney and colleagues (2000) have provided evidence that not only is PtdIns(3)P synthesized in the nuclear matrix, but PtdIns 3-kinase colocalizes with active nucleolar transcription sites in soybean. These data argue against the classical role of class III PtdIns 3-kinases in vesicle-mediated transport to the vacuole/lysozome, and suggest a previously unsuspected function for these enzymes. In contrast, Kim and colleagues (2001) present compelling data that supports the established PtdIns 3-kinase signaling paradigm. Using the endosome binding domain (EBD) of human EEA1 (which specifically binds PtdIns(3)P) fused to green fluorescent protein, the authors demonstrate that PtdIns(3)P is initially synthesized at the TGN, subsequently trafficked to the prevacuolar compartment, and then delivered to the lumen of the central vacuole in *Arabidopsis* protoplasts. Surprisingly, overexpression of a PtdIns 4-kinase β deletion mutant inhibited vacuolar trafficking of GFP-EBD, suggesting the involvement of PtdIns(4)P in vacuolar transport. In addition, it was determined that exposing the protoplasts to the PI 3-kinase inhibitor wortmannin causes missorting of the NTPP-containing vacuolar protein, sporamin. This last result contradicts a previously published report demonstrating that trafficking of sporamin to the central vacuole in tobacco cells is unaffected by wortmannin (Matsuoka et al., 1995). Whether this apparent paradox is a result of species-specific sorting machinery will need to be addressed in future studies.

The Role of AtVps34p in Vacuolar Protein Transport

The seemingly enigmatic function of the plant PtdIns 3-kinase stimulated us to investigate both the subcellular distribution of this enzyme and its putative role in the plant cell. To address the initial question we fused the gene encoding enhanced green fluorescent protein (EGFP) to *AtVPS34*, stably transformed this construct into mature *Arabidopsis* plants and analyzed the intracellular location of the EGFP-AtVps34p by confocal microscopy. To address the latter question we utilized high performance liquid chromatography (HPLC) and immunohistochemical techniques to correlate *in vivo* PtdIns(3)P levels with delivery of a marker protein to the plant cell vacuole. Our data demonstrates that AtVps34p is predominantly a cytosolic protein, although a portion appears to be associated with discreet intracellular locations. Furthermore, we provide evidence that reduced cellular levels of PtdIns(3)P are correlated with missorting of a vacuolar marker protein. In concert, these data support the notion that the PtdIns 3-kinase encoded by *AtVPS34* is involved in regulating the delivery of proteins to the plant cell vacuole.

CHAPTER II

METHODS

Organisms and Media

Arabidopsis thaliana ecotype Columbia was grown in 0.5 X Murashige and Skoog (MS; Sigma, St. Louis, MO) medium containing 1X B5 vitamins. Sterile Erlenmeyer flasks containing *Arabidopsis* seeds were vernalized for two d and then placed on a gyratory shaker at 80 rpm in a growth chamber set at a 14 hr day (22°C)/10 hr night (18°C) cycle. Alternatively, seeds were spread onto agar plates containing 0.5 X MS medium and 1 X B5 vitamins, vernalized for two days and then grown on the previously described day/night cycle. All transgenic seeds were grown in the presence of 50 µg/ml kanamycin.

Immunofluorescence Staining

Arabidopsis thaliana root tips cells were isolated as described previously (Paris et al., 1996). Briefly, root tips from approximately 10-day-old *Arabidopsis* plantlets were cut and fixed for at least 1 hr in 3.7% formaldehyde in PHEM (60 mM Pipes, 25mM Hepes, 10 mM EDTA, 2mM $MgCl_2$) buffer, pH 6.9. The fixed root tips were then treated with 3% cellulysin (Calbiochem, La Jolla, CA) for 30 min to partially degrade the cell wall. Individual cells were then released by gently squeezing the root tips between two coverslips previously coated with 1% 2-aminopropyltriethoxysilane (Sigma, St. Louis, MO). The isolated cells were then permeabilized with 0.5% Triton X-100 in PHEM buffer for 10 min. The isolated, permeabilized cells were then incubated in blocking

buffer (1X PBS containing 0.5% BSA, 0.5% fish skin gelatin and 0.05% Nonidet P-40) at room temperature for 30 min. The cells were then incubated with primary antibody (rabbit anti-wheat germ agglutinin, 1:500; Sigma) at room temperature for 2 hr. To remove unbound primary antibody, the cells were washed with blocking buffer for 30 min, and then incubated with the secondary antibody (Cy3-conjugated anti-rabbit IgG, 1:200; Jackson ImmunoResearch, West Grove, PA) at room temperature for 1 hr. Following washing with blocking buffer for 30 min to remove any unbound secondary antibody, the cells were washed for 10 min in PBS and then mounted in Pro-Long anti-fade medium (Molecular Probes, Bend, OR).

Laser Scanning Confocal Microscopy and Data Processing

Arabidopsis cells were visualized using a Nikon Diaphot TE300 inverted microscope and a BioRad MRC laser scanning confocal microscope (BioRad, Hercules, CA) in the Keller mount position. A krypton/argon laser producing a 488 nm laser line for excitation, and a bandpass filter (522/32) was used to collect fluorescent emission wavelengths from EGFP.

Phosphoinositide Labeling, Lipid Extraction, and HPLC Anlaysis

Phosphoinositide radiolabeling and HPLC analysis were performed as previously described (Hama et al., 2000). Briefly, approximately 10-day-old *Arabidopsis* plantlets were incubated for 24 hr in 0.5 X MS medium containing 50 μCi/ml *myo*-[2-^{3}H] inositol

(Amersham Pharmacia Biotech, Piscataway, NJ) and 10 μM *myo*-inositol (Sigma, St. Louis, MO). Radiolabeled plantlets were then treated with a final concentration of 0, 10, 30 μM wortmannin in 0.5 X MS medium for 30 min. To completely arrest phosphatase and lipase enzymatic activity, plantlets were incubated in 6% trichloroacetic acid (TCA) at 4°C for 1 hr, followed by washing with excess water to remove contaminating TCA. Plantlets were homogenized in 1 M HCl and methanol/chloroform (1:1) and the organic fraction was aliquoted into a new tube and dried *in vacuo*. The resultant lipids were deacylated by incubation in methylamine reagent (25% methylamine, 42% n-butanol) at 55°C for 50 min and then dried *in vacuo*. The glycerophosphoinositols (gPI) head groups were recovered and purified by a series of extractions in butanol, petroleum ether, ethyl formate (20:4:1) and then dried *in vacuo*. The gPIs were resuspended in sterile water and the radioactivity of each sample determined using a Beckman LS 5801 liquid scintillation counter (Beckman, Fullerton, CA). Radiolabeled gPIs were resolved using anion-exchange high performance liquid chromatography (HPLC). Samples were loaded on a Partisil 10 SAX (4.6 x 250mm) column (Whatman, Clifton, NJ) fitted with an SAX guard column (Phenomenex, Torrance, CA). A Beckman System Gold chromatograph equipped with a UV detector and System Gold Software was used. To monitor column performance, 10 μmoles each of AMP, ADP and ATP were mixed with each sample prior to loading. The gradient conditions were 5 ml of isocratic 10 mM ammonium phosphate (pH 3.8) followed by a linear gradient from 10 mM to 0.7 M in 40 ml of ammonium phosphate (pH 3.8) at a flow rate of 1 ml/min. [^{32}P] gPI(3)P and [^{32}P] gPI(4)P standards were generated using *in vitro* phosphorylation of phosphatidylinositol

with gamma-[^{32}P]ATP (Amersham Phamrmacia Biotech) followed by deacylation as

described previously (Hama et al., 2000).

Generation of Enhanced Green Fluorescent Protein (EGFP) Fusions

Standard molecular biology and recombinant DNA techniques were used unless otherwise stated. The polymerase chain reaction was used to amplify the gene encoding At*VPS34*. The forward primer 5'GAAAGTCGACATGGGTGCGAACGAG 3'was used to introduce a Sal I I restriction site and the reverse primer 5'GAAAGGATCCGTTCAACGCCAGTA3' to introduce a Bam HI restriction site. The 2.4 kb product was cloned into the pGEM T-vector (Promega, Madison, WI) and sequenced by the Utah State University Biotechnology Center to check for accurate amplification of the gene. The pGEM:AtVps34 construct was digested with Sal I and Bam HI and the released fragment was cloned into the pEGFP-C1 vector (Clontech, Palo Alto, CA) at the identical restriction sites. The pEGFP-C1:AtVps34 construct was digested with Nhe I and BamHI to generate a C-terminal fusion of the gene encoding enhanced green fluourescent protein (EGFP) to the gene encoding AtVps34. This 3.3 kb fragment was then cloned into the Xba I and Bgl II restriction sites of the binary vector pGA643 henceforth referred to as 35S-EGFP-AtVps34. This construct was also sequenced to ensure that the appropriate reading frame was maintained across the *GFP:AtVPS34* junction. As a control, pEGFP-C1 was digested with Nhe I and Bam HI to release the gene encoding EGFP, which was subsequently cloned into the Xba I and Bgl II restriction sites of the binary vector pGA643, henceforth referred to as 35S-EGFP.

Plant Transformation

Plant transformations were performed as previously described (Bent, 2000). Briefly, *Agrobacterium tumefaciens* strain LBA4404 harboring the previously described gene constructs (35S-EGFP-AtVps34 or 35S-EGFP) was grown to an OD_{600} of 0.8. The cells were collected by centrifugation and resuspended in infiltration medium (5% sucrose, 0.05% Silwet-L77). *Arabidopsis* plants with secondary bolts and many immature flower clusters were then immersed in the infiltration medium and agitated gently for 20 sec. The inoculated plants were transferred to a high humidity environment for two days and then allowed to grow to seed. The resultant seeds were sterilized (3 min in 95% ethanol, 15 min in 30% bleach, and washed in excess sterile water) and then plated on selection medium (0.5 X MS salts, 1 X B5 vitamins, 1% sucrose, 50 µg/ml kanamycin). The potential transgenic seeds were vernalized for two days to prevent post-harvest dormancy and then grown on the previously described day/night cycle. After approximately 10 d of growth, transformants were easily identifiable by dark green shoot tissue and well developed roots that extended significantly into the selection media. Plants capable of growing in the presence of kanamycin were then transferred to soil and brought to seed. A portion of the resultant seeds was germinated as previously described, and the pertinent tissue (root or shoot) was mounted on glass slides for observation on the confocal microscope. Another portion of the seeds was used for production of the F_1 generation of transformed plants.

CHAPTER III

RESULTS

The *Arabidopsis* PtdIns 3-kinase AtVps34p–A Cytosolic Protein That Associates with Specific Intracellular Compartments

To investigate the intracellular location of AtVps34p we fused the gene encoding the bioluminescent reporter molecule enhanced green fluorescent protein (EGFP) to *AtVPS34* (Figure 4A). This construct was introduced into approximately 4-week-old *Arabidopsis* plants by the floral dip method (Bent, 2000). As a control, we also introduced the gene encoding EGFP (sans *AtVPS34*) into *Arabidopsis* plants (Figure 4B). This latter construct was critical for subsequent data interpretation as it permitted us to assess the possibility that EGFP might arbitrarily localize AtVps34p.

To visualize the subcellular distribution of both EGFP-AtVps34p and EGFP we imaged living cells from a variety of tissue types using confocal microscopy. Initially, the most striking observation was the variable degree of EGFP expression; even in a single root tip we noticed significant differences in cell-to-cell expression levels. While the ultimate cause of this phenomenon is unknown, variability in ectopic expression may in fact result from cosuppression/post transcriptional gene silencing, as has been previously documented in other studies (Hong et al., 1999). As a control, we also imaged wild type *Arabidopsis* plants (that do not express EGFP) and were unable to detect any appreciable fluorescent signal (data not shown).

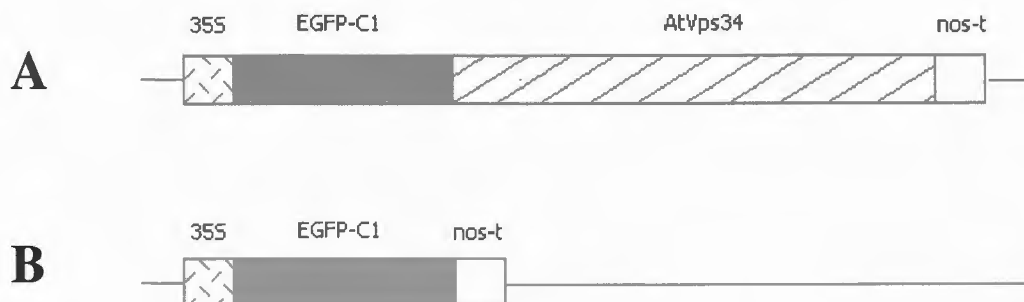

Figure 4. Schematic Representation of the Gene Constructs Utilized to Visualize the Subcellular Distribution of AtVps34p.

(A) A C-terminal fusion of the open reading frame (ORF) of the gene encoding enhanced green fluorescent protein (EGFP) to the ORF of AtVps34 was cloned into the binary vector pGA643. **(B)** As a control, the gene encoding EGFP was also cloned into pGA643. Expression of both constructs was driven by the cauliflower mosaic virus (CaMV) 35S promoter.

Representative confocal images of cells near the root tip in plants expressing either EGFP alone or EGFP-AtVps34p are shown in Figure 5 and Figure 6, respectively. In the EGFP control cells, moderate fluorescence was observed in the cytoplasm, and a stronger fluorescent signal in the nucleus. In addition, EGFP seemed to be completely excluded from vacuoles, organelles and the nucleolus, as evidenced by distinct negative staining in these organelles (Figure 5). This observed fluorescent pattern is consistent with previously published reports describing GFP expression in plants (Haselhoff et al., 1997).

In contrast to the EGFP control, cells expressing the EGFP-AtVps34p fusion protein displayed no apparent nuclear fluorescence. However, we did observe a general cytoplasmic signal, as well as discrete intracellular locations that seemed to preferentially accumulate EGFP-AtVps34p (Figure 6A and B). Moreover, a subpopulation of these

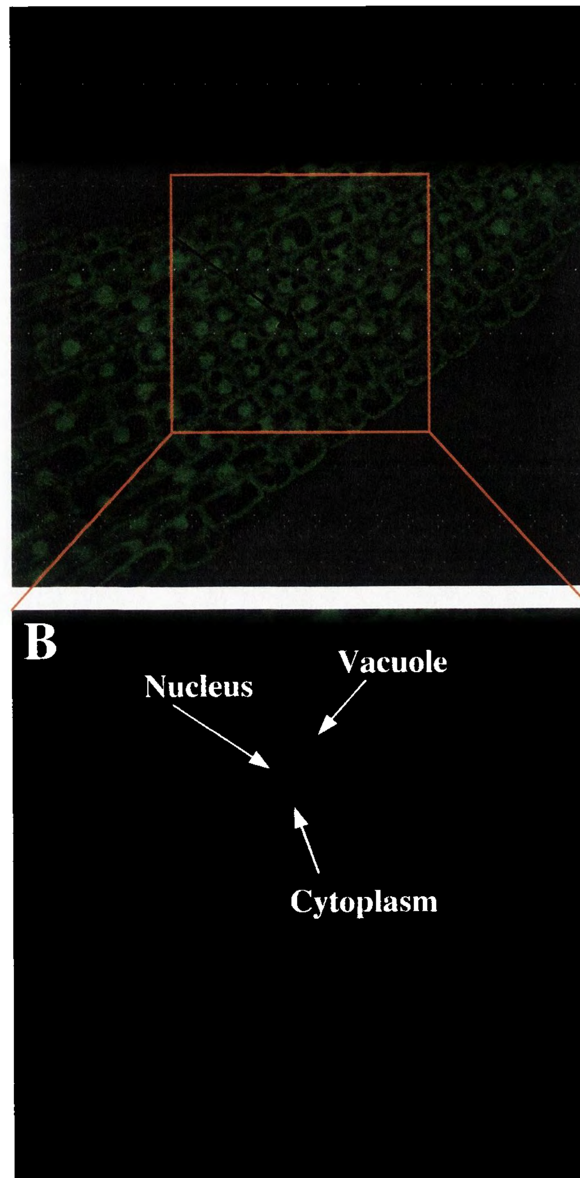

Figure 5. GFP Is Expressed in the Nucleus and Cytoplasm in *Arabidopsis* Root Tip Cells.

Arabidopsis plants stably-transformed with pGA643:EGFP were germinated on agar plates containing kanamycin (50μg/ml) for approximately 10 days. The root tips of these plantlets were then excised and mounted on slides for imaging by confocal microscopy. **(A)** 1X zoom and **(B)** 4X zoom.

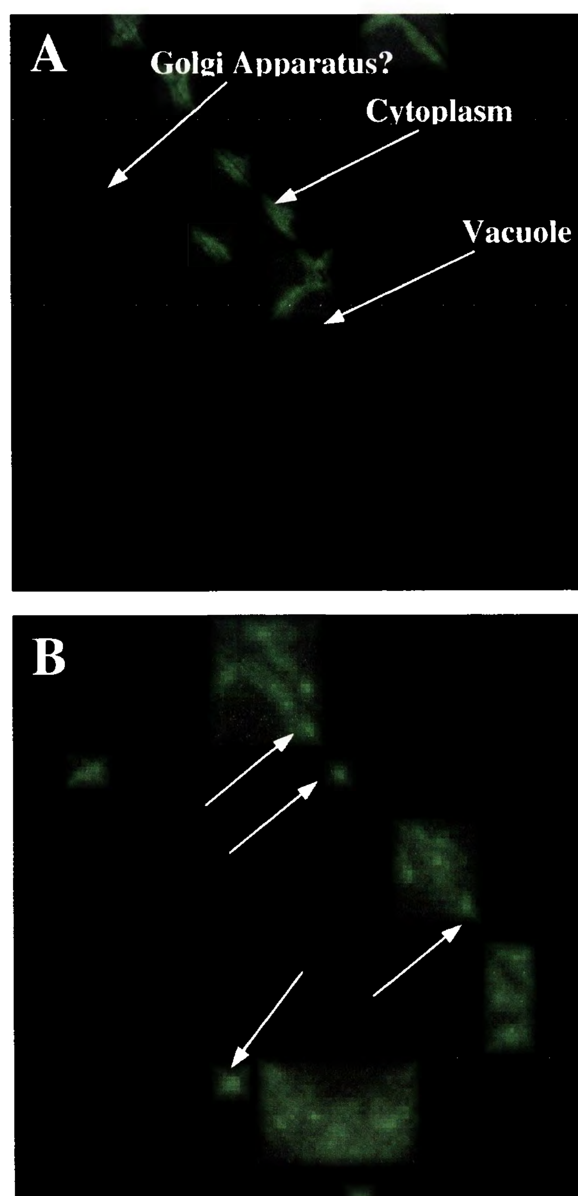

Figure 6. EGFP-AtVps34 Is Expressed in the Cytoplasm in *Arabidopsis* Root Tip Cells.

Arabidopsis plants stably-transformed with pGA643:EGFP:AtVps34 were germinated on agar plates containing kanamycin (50 μg/ml) for approximately 10 days. The root tips of these plantlets were then excised and mounted on slides for imaging. **(A)** and **(B)** are images of the same root tip displayed at increasing magnification to illustrate the distinct fluorescent pattern observed in some cells.

cells displayed an intensely fluorescent punctate pattern that is distinctly reminiscent of the plant Golgi complex (Figure 6B; Satiat-Jeumemaitre and Hawes, 1992; Fitchette et al., 1999).

We also examined guard cells in the leaf tissue, and again, EGFP was consistently expressed at moderate levels in the cytoplasm, at high levels in the nucleus and excluded from other membrane-bound organelles (Figure 7A). In a similar respect, the fluorescent signal from EGFP-AtVps34p was distributed quite uniformly in the cytoplasm, with specific subcellular sites exhibiting an intricate fluorescent pattern. (Figure 7B).

Selective Decrease in Cellular PtdIns(3)P Levels by Wortmannin

Class III PtdIns 3-kinases from different species vary dramatically in their sensitivity to wortmannin. Specifically, the mammalian Vps34p homolog is inhibited at low nanomolar concentrations of the fungal metabolite (Vanhaesebroeck et al., 1997), while the yeast enzyme is apparently unaffected at this concentration (Schu et al., 1993; Woscholski et al., 1994). In plants, *in vitro* kinase assays have demonstrated that wortmannin inhibits both PtdIns 3-kinase and PtdIns 4-kinase activity (Matsuoka et al., 1995), although nothing is currently known regarding wortmannin's effect *in vivo*. To address this issue we labeled approximately 10-day-old *Arabidopsis* plantlets with ^{3}H *myo*-inositol to steady state and incubated them in the absence or presence of different concentrations of wortmannin. We then extracted and deacylated total cellular lipids, and purified the inositol-containing lipid head groups. The resultant head groups were then

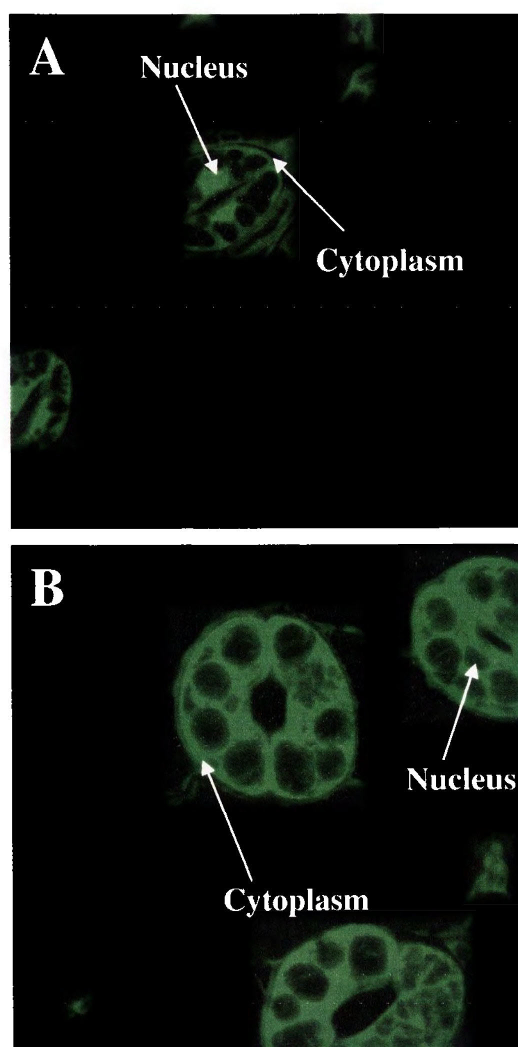

Figure 7. EGFP and EGFP-AtVps34 Expression in *Arabidopsis* Guard Cells.

Arabidopsis plants stably-transformed with **(A)** pGA643:eGFP or **(B)** pGA643:eGFP_AtVps34 were germinated on agar plates containing kanamycin (50 μg/ml) for approximately 10 days. The leaves of these plantlets were then excised, mounted on slides and imaged by confocal microscopy.

separated by anion exchange HPLC. In plants that were not treated with wortmannin, gPI(3)P and gPI(4)P were the most abundant species, although gPI(4)P levels were

dramatically higher than those observed for gPI(3)P (data not shown). This observation demonstrates a dramatic difference in phosphoinositide metabolism between plant cells and yeast cells, which accumulate nearly equivalent amounts of PtdIns(3)P and PtdIns(4)P (Hama et al., 2000).

When plants were incubated in the presence of 10 μM wortmannin, gPI(3)P levels dropped approximately 50%, while gPI(4)P was relatively unaffected (Fig 8). At 30 μM wortmannin, gPI(3)P levels were moderately lower than those observed at 10 μM, but gPI(4)P levels were substantially reduced when compared to 10 μM wortmannin treatment (Figure 8). These findings imply that 10 μM wortmannin can be used to selectively inhibit the *Arabidopsis* PtdIns 3-kinase.

Missorting of Barley Lectin in Transgenic *Arabidopsis*
Plants by Wortmannin

The observation that 10 μM wortmannin can selectively inhibit AtVps34p allowed us to address the possibility that this enzyme has a fundamental role in vesicle-mediated protein transport to the vacuole, as has been described for class III PtdIns 3-kinases in other species (Schu et al., 1993; Brown et al., 1995; Volinia et al., 1995; Siddhanta et al., 1998). To this end we utilized immunofluorescence techniques (Paris et al., 1996) to monitor delivery of a marker protein called barley lectin to the vacuole in stably transformed *Arabidopsis* root tip cells in the absence and presence of the PI 3-kinase inhibitor wortmannin. In the absence of this fungal metabolite, barley lectin was appropriately transported to the vacuoles as evidenced by intensely stained circular structures near the plasma membrane (Figure 9A). In addition, the plasma membrane of

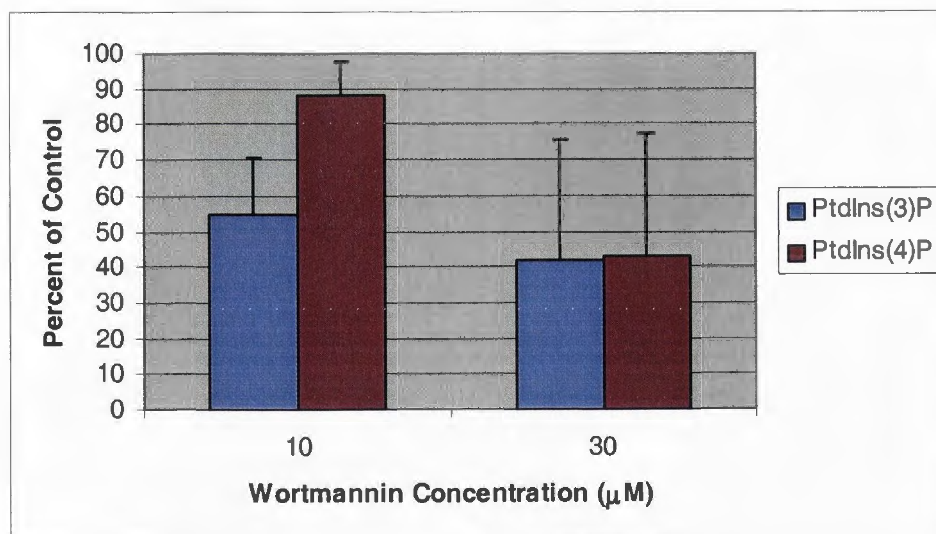

Figure 8. Wortmannin Causes a Selective Decrease in PtdIns(3)P Levels in *Arabidopsis*.

Approximately ten-day-old *Arabidopsis* plantlets were incubated for 24 hr in the presence of ^{3}H *myo*-inositol and then treated with 0, 10 or 30 μM wortmannin for 30 min. Post-treatment, the plantlets were homogenized in the presence of chloroform/methanol/HCl to isolate total cellular lipids. This organic fraction was deacylated using methylamine and the resultant lipid head groups purified by butanol extraction. The isolated products were then separated by anion exchange HPLC. Total counts in the peaks corresponding to PtdIns(3)P and PtdIns(4)P (of three independent experiments) were averaged, normalized against the control (mock treatment) and plotted with standard deviation.

these cells also displayed a fluorescent signal, indicating that the anti-wheat germ agglutinin antibody recognizes some moiety present on this membrane. The fluorescent pattern described for transgenic *Arabidopsis* root tip cells expressing barley lectin in this study has been previously observed for endogenous barley lectin in barley root tip cells (Paris et al., 1996).

At 10 μM wortmannin barley lectin was not delivered to the vacuole, but apparently transported in the default route of the secretory pathway (Figure 1 route 5) to

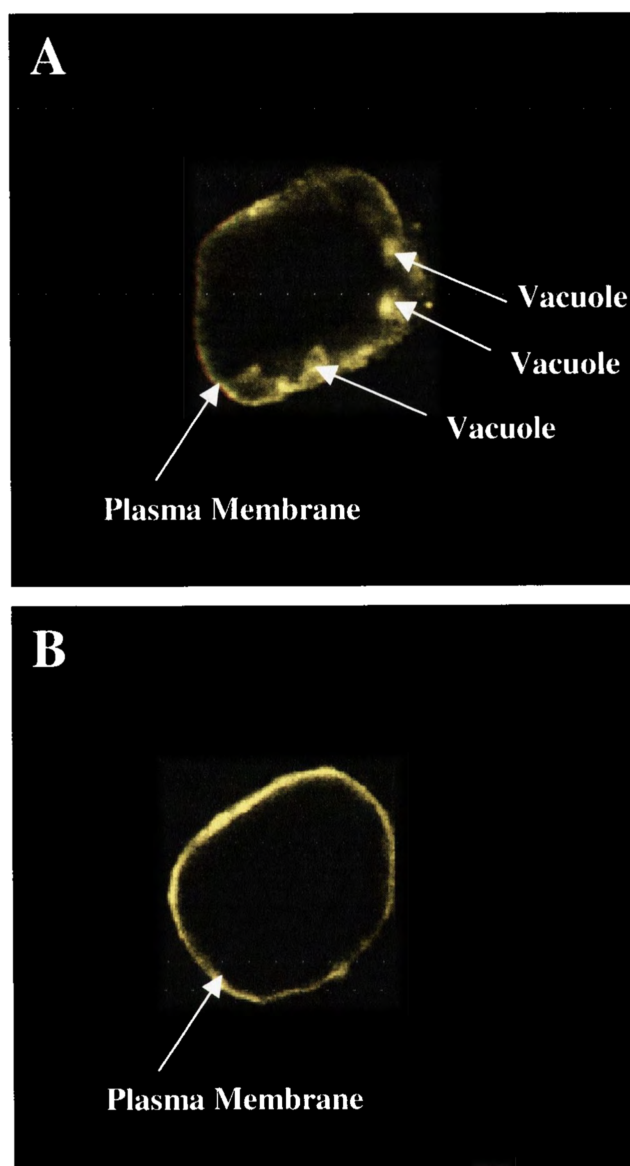

Figure 9. Wortmannin Causes Missorting of Barley Lectin in Transgenic *Arabidopsis* Root Tip Cells.

Approximately 10-day-old *Arabidopsis* plants were incubated in the **(A)** absence or **(B)** presence of wortmannin for 30 min and then processed as described in *Methods.*

the plasma membrane for egress from the cell. Characteristically, cells from wortmannin-treated plants exhibited no intracellular fluorescence, although a signal at the

plasma membrane persisted (Figure 9B). It is critical to note that not all of the wortmannin-treated cells displayed this phenotype. In fact, some cells closely resemble those described in Figure 9A. An obvious explanation of this occurrence revolves around the ability of wortmannin to penetrate each cell. That is, was each individual root tip cell exposed to the same effective concentration of wortmannin? While this issue would be difficult to address *in vivo*, we would speculate that the answer is no. Importantly, however, the visual phenotype that we associate with barley lectin missorting (see Figure 9A) was not observed during evaluation of cells that were incubated in the absence of wortmannin, suggesting that the cellular egress of barley lectin was due to treatment with this pharmacological agent. Importantly, the observation that 10 μM wortmannin (along with inhibiting vacuolar deposition of barley lectin), significantly perturbs the synthesis of PtdIns(3)P but not PtdIns(4)P suggests that PtdIns(3)P is involved in vesicle-mediated protein transport to the vacuole in plant cells.

PtdIns(3)P-NBD Localization to Endosomal/Vacuolar Structures in *Arabidopsis* Roots

In an effort to investigate the potential role of PtdIns(3)P in vacuolar protein transport we attempted to localize this phosphoinositide in *Arabidopsis* root tip cells using a fluorophore-conjugated synthetic analog (PtdIns(3)P-NBD). PtdIns(3)P-NBD was delivered to root tip cells by an established method that does not involve endocytosis (Ozaki et al., 2000) and then visualized using confocal microscopy. Analysis of these images demonstrated considerable cell-to-cell variation in uptake efficiency; some cells fluoresced brightly and others remained perpetually dark.

The cells that were efficiently loaded with PtdIns(3)P-NBD exhibited a characteristic fluorescent pattern. Specifically, we observed an intense signal not only from the membrane of small, circular structures that resembled vesicles or endosomes (Figure 10), but also from a membrane that delimited the entire cell (and possibly corresponded to the tonoplast) (Figure 10). Of particular interest was the dynamic nature of these structures, which seemed to bud from and fuse with one another (Figure 10). Perhaps most intriguingly, the fluorescent signal of PtdIns(3)P-NBD disappeared over time (data not shown), implying that this phosphoinositide analog might be degraded in manner similar to endogenous PtdIns(3)P.

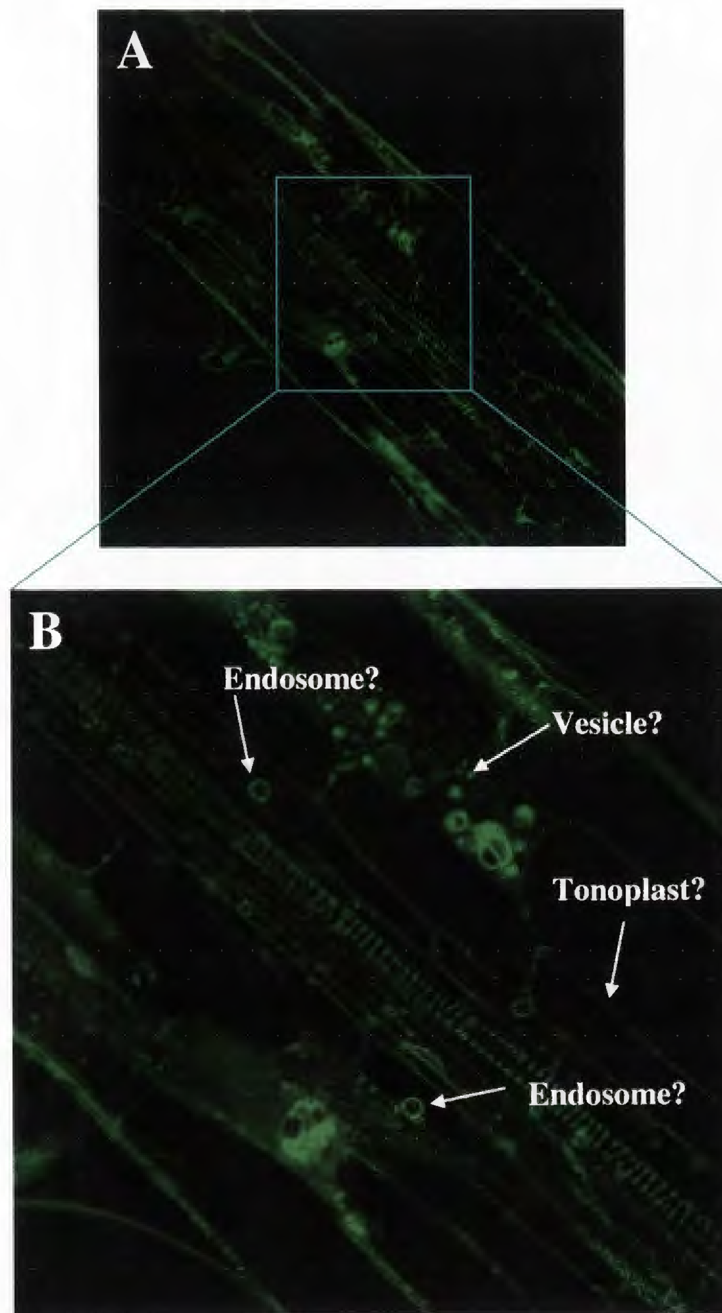

Figure 10. PtdIns(3)P-NBD Localization in *Arabidopsis* Root Tip Cells.

(A) 1X zoom and **(B)** 4X zoom of confocal images of NBD-PtdIns(3)P delivered to wild type *Arabidopsis* roots.

CHAPTER IV

DISCUSSION

AtVps34p Displaying a Generally Cytosolic Distribution Pattern

To investigate the function of AtVps34p, we initially examined the subcellular location of the *Arabidopsis* PtdIns 3-kinase by constructing a fusion between the gene encoding EGFP and At*VPS34*. While inspection of root tips cells in plants that were transformed with this gene fusion revealed a predominantly cytosolic fluorescent signal, we also observed accumulation of EGFP-AtVps34p at discrete intracellular locations that may correspond to the Golgi and/or endosomal compartment. The possible association of the *Arabidopsis* PtdIns 3-kinase with these organelles is corroborated by studies in both yeast (Stack et al., 1993) and mammalian (Siddhanta et al., 1998; Domin et al., 2000) cells and is consistent with a potential role for PtdIns 3-kinase in both biosynthetic and endocytic transport in plant cells. However, our findings do oppose a recent report demonstrating that a class III PtdIns 3-kinase is restricted to nuclear transcription sites in soybean (Bunney et al., 2000). Moreover, the conclusions rendered by Bunney and colleagues (2000) contradict a previous study of the soybean Vps34p homolog, which describes a functional involvement in membrane proliferation (Hong and Verma, 1994). At present, it is unknown whether these antithetical data represent a species-specific phenomenon or are a result of experimental artifact. Although there is an increasing body of evidence supporting nuclear phosphoinositide signal transduction (Boronenkov et al., 1998; Tanaka et al., 1999; York et al., 1999), we agree with the amalgam of

experimental data supporting the involvement of PtdIns 3-kinases in vesicle-mediated protein transport in eukaryotic cells (Schu et al., 1993; Brown et al., 1995; Volinia et al., 1995; Siddhanta et al., 1998).

The observation that EGFP-AtVps34p is a cytosolic fusion protein is not surprising when considering that only 50% of the yeast Vps34p is found in a pelletable membrane fraction in wild type cells, and 90% of Vps34p is found in the cytosol when *VPS34* is overexpressed (Stack et al., 1993; reviewed in DeWald et al., 1997). The molecular basis for these observations is the requirement of the *VPS15* gene product (Vps15p) for membrane association and activation of Vps34p (reviewed in DeWald et al., 1997). In yeast cells that overexpress Vps34p, endogenous Vps15p molecules are saturated with their cognate partner and unable to translocate the majority of overexpressed Vps34p to the appropriate membrane domain. An experiment that would evaluate the plausibility of this explanation is to coexpress untagged *Arabidopsis* Vps15p and EGFP-tagged AtVps34p and determine whether the preponderance of Vps34p is membrane associated. The *Arabidopsis* Vps15p homolog has not yet been cloned, although we have recently uncovered a candidate cDNA via the *Arabidopsis* genome sequencing initiative (Jones and DeWald, unpublished observations).

An alternative explanation for the cytosolic distribution of AtVps34p is that EGFP perturbs the three-dimensional structure of AtVps34p and prevents the fusion protein from appropriately interacting with endogenous AtVps15p. While investigation of this hypothesis is presently impossible (as the At*VPS15* gene has not yet been cloned), it deserves attention in future studies. The possibility that EGFP tagging prohibits

functional interaction between molecular partners is a potential pitfall of using this method.

Correlation of Missorting of Barley Lectin with Reduced Levels of Cellular PtdIns(3)P

Both genetic and pharmacological studies have conclusively demonstrated that PtdIns 3-kinase is involved in biosynthetic protein transport in a variety of cell types (Schu et al., 1993; Brown et al., 1995; Matsouka et al., 1995; Kim et al., 2001). In this study we used a combination of biochemical and immunofluorescent techniques to demonstrate that PtdIns 3-kinase (and its lipid product PtdIns(3)P) is involved in Golgi-to-vacuole protein transport in *Arabidopsis*. Specifically, HPLC analysis, in conjunction with immunolabeling experiments revealed that the vacuolar marker protein barley lectin is secreted from the cell (instead of being transported to the vacuole) at a concentration of wortmannin that reduces the cellular level of PtdIns(3)P by approximately 50%, but does not dramatically alter PtdIns(4)P. In this context, a recent report providing evidence that sweet potato sporamin (expressed in *Arabidopsis* protoplasts) is missorted in the presence of wortmannin (Kim et al., 2001), is significant, and raises several issues. First, the findings presented by Kim and colleagues (2001) dispute a study that previously demonstrated that vacuolar transport of BL, but not sporamin is perturbed by wortmannin in tobacco BY-2 cells (Matsuoka et al., 1995). This difference underscores the possibility that unique sorting machinery exists in these two species.

Second, the data presented in our study, juxtaposed with that from Kim and colleagues (2001), seems to further complicate the "two pathway" paradigm for vacuolar

transport in plant cells. Specifically, these reports show that wortmannin induced missorting of both a CTPP- (barley lectin) and NTPP-containing (sporamin) protein, suggesting that, at least in *Arabidopsis*, these two proteins are transported to the vacuole via the same pathway. This prospect may be improbable from a mechanistic standpoint, however, when considering that pro-sporamin can bind to a putative plant vacuolar targeting receptor, but pro-barley lectin cannot (Kirsch et al., 1994). Alternatively, it is feasible that barley lectin and sporamin could be delivered to the vacuole in pathways that are similar in their relative sensitivity to wortmannin, but dissimilar in their respective machineries (i.e., sorting receptors).

At issue here as well may be cell maturity. We used protoplasts isolated from the terminal portion of *Arabidopsis* root tips (which represent undifferentiated cells and contain many small vacuoles (Paris et al., 1996) to assay barley lectin localization, while Kim and colleagues (2001) used protoplasts that contained a central vacuole (and arguably correspond to differentiated cells) to observe sporamin trafficking.

The difficulty in resolving these findings exposes an obvious consequence of utilizing heterologous expression systems to study vacuolar protein trafficking. This complication might be avoided by using an endogenous protein as a marker, as has been achieved in both yeast (carboxypeptidase Y; Schu et al., 1993) and mammalian (cathepsin D; Brown et al., 1995) cells. The obvious advantage of this approach is that the marker protein is transported by the cell's intrinsic trafficking machinery.

Another conspicuous conclusion revolves around the involvement of PtdIns(4)P in PtdIns(3)P-dependent trafficking to the vacuole (Kim et al., 2001). The authors used both a PtdIns 4-kinase β deletion mutant and wortmannin (10 μM) to retard delivery of

GFP-EBD to the tonoplast, and thereby assume that PtdIns(4)P is somehow helping regulate this process. In addition, they used wortmannin (5 μM) to inhibit vacuolar deposition of sporamin. These data suggest that both PtdIns 3-kinase and PtdIns 4-kinase activity are requisite for accurate and efficient transport of proteins to the vacuole, although our data suggests otherwise. Specifically, HPLC analysis of inositol-containing lipid head groups isolated from plants incubated with 10 μM wortmannin demonstrate that gPI(4)P levels are nearly indistinguishable from those observed in untreated plants, while gPI(3)P levels are substantially reduced. These data seem to imply that PtdIns(3)P is the PtdIns monophosphate species necessary for vesicle-mediated transport to the vacuole in *Arabidopsis* and PtdIns(4)P is involved in ancillary intracellular processes. This hypothesis is supported by studies in both yeast and mammalian systems which provide evidence that PtdIns(3)P regulates protein traffic to the lysosome/vacuole (Schu et al., 1993; Brown et al., 1995), while PtdIns(4)P regulates protein secretion (Wiedemann et al., 1996; Hama et al., 2000). However, two possibilities exist which might explain the purported involvement of PtdIns(4)P in PtdIns(3)P-dependent trafficking to the vacuole. First, wortmannin might have improved access to the target enzymes within *Arabidopsis* protoplasts, and accordingly, PtdIns(4)P levels would be lower than those observed in our study. Second, it is feasible that seemingly insignificant reductions in the total PtdIns(4)P pool might actually represent an important depletion of spatially restricted PtdIns(4)P. That is, if the observed 15% loss of PtdIns(4)P occurred in specific microdomains of the Golgi complex, where, for example, protein cargo is packaged in phospholipid-containing vesicles for transit to the vacuole, a slight global reduction in PtdIns(4)P could have a profound effect on this process.

PtdIns(3)P-NBD Found in Intracellular

Membranes

A number of recent reports have revealed the functional significance of PtdIns(3)P in membrane transport (reviewed in Odorizzi et al., 2000). PtdIns(3)P regulates multiple steps in both secretory and endocytic trafficking by specifically recruiting a set of downstream effector molecules to the appropriate intracellular membrane. A PtdIns(3)P binding protein in the endocytic pathway is the early endosomal autoantigen 1 (EEA1). This peripheral membrane protein has been demonstrated to interact with activated Rab5 GTPase and stimulate homotypic endosome fusion in *vitro* (Simonsen et al., 1998). Consistent with a role as downstream effector of PtdIns 3-kinase, the endosomal localization of EEA1 is perturbed by wortmannin treatment and EEA1 copurifies with liposomes that contain PtdIns(3)P but not other phosphorylated PtdIns species (Simonsen et al., 1998). EEA1's intrinsic lipid binding activity is due to a C-terminal cysteine-rich region called the FYVE domain (for Fab1, YGLO23, Vps27, and EEA1) (Mu et al., 1995; Stenmark et al., 1996). FVYE domains have been identified in several other mammalian and yeast proteins, including Hrs (Komada and Soriano, 1999), Vps27p (Wurmser et al., 1999), Vac1p (Tall et al., 1999), and Fab1p (Gary et al., 1998). Interestingly, all of these proteins help regulate distinct stages in biosynthetic/endocytic membrane transport.

Recently, the endosome binding domain of EEA1 has been used to determine the subcellular location of PtdIns(3)P in *Arabidopsis* protoplasts (Kim et al., 2001). Kim et

al. (2001) utilized an endosome binding domain-GFP fusion construct to monitor PtdIns(3)P trafficking from the TGN to the lumen of the central vacuole. In the present study, we employed a fluorescent phosphoinositide analog to evaluate the intracellular distribution of PtdIns(3)P. In these experiments we observed intense fluorescence from intracellular structures that resembled endosomes or vesicles, as well as a moderate signal from a membrane possibly corresponding to the tonoplast. Moreover, intracellular PtdIns(3)P-C_6-C_6-NBD behaved in a dynamic manner, and fluorescence associated with this molecule eventually disappeared. These data are consistent with the previously published findings of Kim and colleagues (2001), and provide additional support for the notion that PtdIns(3)P may play an important role in membrane transport in plants.

At present, 17 FYVE domain-containing proteins have been identified in the *Arabidopsis* genome, suggesting the importance of PtdIns(3)P in plant cell biology. Moreover, a plant 3-phosphoinositide-dependent protein kinase-1 (AtPDK1) homolog that contains a pleckstrin homology domain has been cloned (Deak et al., 1999). A GST-AtPDK1 fusion protein has been shown to bind phosphatidic acid, PtdIns(3)P, PtdIns(3,4)P_2, PtdIns(3,4,5)P_3 and to a lesser degree, PtdIns(4)P and PtdIns(4,5)P_2 (Deak et al., 1999). These data are interesting since PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 have not yet been identified in *Arabidopsis* (Jones, Torabinejad and DeWald, unpublished observations), suggesting that AtPDK1 may function downstream of PtdIns(3)P. Whether this protein has a heretofore unsuspected role in vacuolar protein transport in plant cells remains to be seen.

Perspectives

In this study we have provided evidence that the *Arabidopsis* PtdIns 3-kinase AtVps34p and its lipid product PtdIns(3)P are involved in vesicle-mediated protein transport to the plant cell vacuole. In particular, our data suggests that AtVps34p synthesizes PtdIns(3)P on an intracellular membrane likely corresponding to the Golgi or endosomal compartment. Transport vesicles containing membrane-restricted PtdIns(3)P bud from this organelle and travel through the cytosol, eventually fusing with the tonoplast and releasing their lumenal contents into the vacuole. It is also possible that TVs fuse with an intermediate compartment (such as the pre-vacuolar compartment) prior to vacuolar protein deposition, as has been suggested for yeast (Stack et al., 1993) and *Arabidopsis* protoplasts (Kim et al., 2001). In any regard, once transport vesicles fuse with the vacuole, PtdIns(3)P becomes a tonoplast-restricted molecule and presumably awaits further modification or degradation. While in plants the mechanistic details of these processes are purely speculative, PtdIns(3)P turnover in yeast has been well documented. Currently, two discrete pathways have been defined which seem to terminate signaling through PtdIns(3)P. The first involves delivery of PtdIns(3)P to the vacuole (for degradation by vacuolar hydrolases) via *VAM3/YPT7*-dependent vesicle trafficking events (Wurmser and Emr, 1998). Mutants with either compromised vacuolar hydrolytic activity or deletion of *VAM3/YPT7* exhibit a several-fold increase in cellular PtdIns(3)P levels, indicating that this may be the principal turnover mechanism in yeast (Wurmser and Emr, 1998). Alternatively, PtdIns(3)P can be phosphorylated at the D-5 position of the inositol ring by Fab1p, a yeast PtdIns(3)P 5-kinase that has been

implicated in vacuolar homeostasis (Gary et al., 1998) and protein sorting in the multivesicular body (Odorizzi et al., 1998). While the *Arabidopsis* Fab1p homolog has not been cloned, a candidate cDNA has been identified in the *Arabidopsis* genome (Jones and DeWald, unpublished observations).

While this study has provided evidence linking PtdIns(3)P to vesicle-mediated protein transport to the vacuole, future studies will need to clarify and expand upon these observations. Specific issues that must be addressed include, but are not limited to the following: Co-localization of EGFP-AtVps34p expression with specific endomembrane markers. This goal might be efficiently accomplished using both subcellular fractionation, and indirect immunofluorescence techniques. Identification of the precise subcellular location of AtVps34p is critical in evaluating the ultimate function of this enzyme in plant cells. It would also be informative to co-localize barley lectin and PtdIns(3)P in *Arabidopsis* root tip cells. This experiment would lend further credence to the idea that PtdIns(3)P is fundamentally involved in transport of barley lectin to the vacuole. Finally, corroboration of wortmannin-induced missorting of barley lectin, perhaps through pulse-chase analysis, would likewise be useful.

On a more global scale, the plant intracellular trafficking community must begin to clone the genes that are involved in PtdIns(3)P-dependent transport of proteins from the Golgi compartment to the vacuole. The significant strides made in defining this pathway in yeast should pave the way for rapid progress in plants. However, a recent review has chronicled the subtle differences between yeast and plant vesicle trafficking machinery (Bassham and Raikhel, 2000), and in this respect, investigators of plant vacuolar protein transport should be aware that "plants are not just green yeast."

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