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dissertation entitled

CHARACTERIZATION OF A FAMILY OF VACUOLAR SORTING RECEPTORS IN ARABIDOPSIS THALIANA

presented by

EMILY AVILA-TEEGUARDEN

has been accepted towards fulfillment
of the requirements for the

Doctoral degree in Cell and Molecular Biology

Natasha V Raikhel
Major Professor's Signature

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Date

CHARACTERIZATION OF A FAMILY OF VACUOLAR SORTING RECEPTORS IN ARABIDOPSIS THALIANA

By

Emily Avila-Teeguarden

A DISSERTATION

Submitted to
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DOCTOR OF PHILOSOPHY

Cell and Molecular Biology Program

2004

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ABSTRACT

CHARACTERIZATION OF THE FAMILY OF VACUOLAR SORTING RECEPTORS IN *ARABIDOPSIS THALIANA*

By

Emily Avila-Teeguarden

In plant cells, soluble proteins delivered to the vacuole via the endomembrane system contain a vacuolar sorting signal (VSS). Several types of VSSs have been identified in plants. One class of VSS is the N-terminal propeptide (NTPP) that is cleaved from the mature protein and contains a conserved peptide motif required for vacuolar sorting. A putative vacuolar sorting receptor (VSR) for NTPP-containing proteins is the *Arabidopsis* vacuolar sorting receptor 1 (AtVSR1; formerly AtELP). AtVSR1 is a type I transmembrane protein with a protease-associated domain and three cysteine-rich EGF repeats. Plant VSRs interact with NTPP-containing proteins in a sequence-specific manner to direct their delivery to lytic vacuoles via a prevacuolar compartment. *AtVSR1* has six homologues in the *Arabidopsis* genome. Expression of all members of this gene family was detected in various plant tissues. To understand the specific roles played by each of these proteins, I transformed *promoter::GUS* fusion constructs into *Arabidopsis* to determine the cell type-specific expression pattern of each gene. From this approach, I determined that many of the *AtVSRs* were expressed in cell type specific expression patterns. For example, the *AtVSR3* gene was specifically expressed in the guard cells of true leaves. Other genes, such as *AtVSR1*, were expressed throughout the vascular tissue as well as in developing and mature embryos. Two genes, *AtVSR2* and *AtVSR5*, showed much broader expression patterns than RT-PCR results

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indicated. These results are likely due to the presence of negative regulatory elements in the introns or 3'UTR of the genes that were absent from the *promoter::GUS* fusions.

Reverse genetics and biochemical approaches were also used to understand the collective and individual functions of these proteins. An antisense approach was used to post-transcriptionally silence the entire *AtVSR* gene family. No plants were obtained that silenced expression from all the AtVSRs. Furthermore, there was very low heritability of the AtVSR silencing. These results indicated that at least some amount of AtVSR protein is essential to plant growth and development. Transgenic plants that had the lowest levels of AtVSR expression showed severe defects in root and shoot gravitropism, defects in leaf and flower development, and produced very few seeds. The pleiotropic effects of silencing the AtVSR gene family indicated that these genes play numerous and varied roles in plant development. To determine the functions of individual AtVSRs, we took advantage of other reverse genetic strategies. Specifically, RNA interference of the *AtVSR3* gene produced plants that accumulated anthocyanins in the cotyledons and were smaller than wildtype seedlings. Preliminary results also suggested that the stomata may not respond to signal transduction pathways that cause stomata to close. Two independent knockout lines of AtVSR7 produced very small plants. Overall, these results demonstrated that plant VSRs function in very specific pathways which has not been shown for other eukaryotes. Other projects presented here relate to vacuolar biogenesis. Specifically, I initiated a high-throughput confocal microscopy screen for mutants that did not form vacuoles properly and a proteomics survey of plant cell vacuoles. These projects have helped Dr. Raikhel's lab as well as the scientific community at large move into a high-throughput systems biology approach to plant science.

To The Memory of Alberto Luis Avila (1931-1987)

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ACKNOWLEDGMENTS

I must first thank my advisor and mentor, Dr. Natasha Raikhel. Natasha has been more of a mother figure to me than anyone in my life. She has taught me by example how to acknowledge and deal with the challenges in my life. I will always be grateful to her for giving me the strength I needed to change my life for the better.

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I must also thank Dr. Marci Surpin for all of her help and support both professionally and personally. Also, I specially thank Dr. Marci Surpin, Dr. Glenn Hicks, and Dr. Clay Carter for critical reading of this thesis. All of the past and present members of the Raikhel lab have been excellent teachers, colleagues, and friends. I would like to specifically thank Dr. Sharif Ahmed, who helped me continue his work on vacuolar sorting receptors. I would also like to thank Dr. Thomas Girke and Dr. Curt Wilkerson for their help with bioinformatics. Much of this work could not have been done without them. Dr. Natasha Raikhel, Dr. Shirley Owen, Dr. Joanne Whallon, Dr. David Carter, Cathy Ecker and Dr. Marguerite (Rita) Varagona all encouraged my interest in microscopy. On a personal level, I am grateful to Rita for taking me under her wing and encouraging me.

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Gene Trap and Enhancer Trap lines with me as well as their knowledge about them. I also thank the Salk Institute Genomic Analysis Laboratory for providing the Sequence-indexed Arabidopsis T-DNA Insertion Mutants. Dr. Vicki Chandler and Dr. Robert Jorgensen kindly sent the dsRNA vector. Dr. Chris Somerville generously provided the 35S::GFP:Δ-TIP line. Dr. Jian-Kang Zhu generously allowed me to use the thermal camera in his laboratory. Dr. Louis King helped me try to sort vacuoles by FACS. Syngenta kindly analyzed my vacuolar protein extracts.

On a personal level, I am thankful for my dearest husband, Thomas Teeguarden. His never-ending patience and faith in me is amazing. He is definitely my inspiration to become a better person. I am also extremely grateful to Carmen, Scherrie, Karen, Ginny, Cathy, Sharon, Christine, Robbie, and Margaret. As a survivor of child abuse, I was convinced that nobody, including myself, would ever understand the long-term effects I faced, except for this incredible group. There will always be a special place in my heart for these brave women. In this context, I must also thank my sister, Elaine, for calmly waiting 15 years and for being so prepared to immediately help me when I was ready to start to heal.

Finally, this work was supported in part by the MSU-DOE Plant Research Laboratory and a Graduate Research Fellowship from the National Science Foundation.

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α-TIP
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ABA
ABC
agg
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BP-80
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cDNA
CPY
CTPP
DNA
DOF
DPBF
dsRNA
DV
EGF
ELP
EMS
ER
EST
FACS
GA
GFP
GUS
IGFII
KLH
KV
LV
M-6-P
MRP
MPR
mRNA
MVB
NASC

LIST OF ABBREVIATIONS

α-TIP	=Alpha Tonoplast Intrinsic Protein
γ-TIP	=Gamma Tonoplast Intrinsic Protein
Δ-TIP	=Delta Tonoplast Intrinsic Protein
ABA	=Abscisic Acid
ABC	=ATP-Binding Cassette
agg	=*aggregates of GFP*
ALP	=Alkaline Phosphatase
AP	=Adaptor Protein
ARE	=AU-Rich Element
ATP	=Adenosine Triphosphate
BP-80	=Binding Protein of 80kDa
bub	=*bubble-bath*
CCV	=Clathrin-Coated Vesicle
CD-MPR	=Cation-Dependent Mannose-6-Phosphate Receptor
cDNA	=Complementary Deoxyribonucleic Acid
CPY	=Carboxypeptidase Y
CTPP	=C-Terminal Propeptide
DNA	=Deoxyribonucleic Acid
DOF	=DNA-Binding With One Finger
DPBF	=Dc3 Promoter-Binding Factor
dsRNA	=Double-Stranded Ribonucleic Acid
DV	=Dense Vesicle
EGF	=Epidermal Growth Factor
ELP	=Epidermal Growth Factor Receptor-Like Protein
EMS	=Ethylmethane Sulfonate
ER	=Endoplasmic Reticulum
EST	=Expressed Sequence Tag
FACS	=Fluorescence Assisted Cell Sorter
GA	=Gibberellic Acid
GFP	=Green Fluorescent Protein
GUS	=β-Glucuronidase
IGFII	=Insulin-Like Growth Factor II
KLH	=Keyhole Limpet Hemocyanin
KV	=KDEL Vesicle
LV	=Lytic Vacuole
M-6-P	=Mannose-6-Phosphate
MRP	=Multidrug Resistance-associated Protein
MPR	=Mannose-6-Phosphate Receptor
mRNA	=Messenger Ribonucleic Acid
MVB	=Multivesicular Body
NASC	=Nottingham Arabidopsis Stock Centre

NTPP
NtSyr1
OSM1
PA
PAC
PBS
PCR
PIN
PPV
PrA
PrB
PSV
PTGS
PV72
PVC
RER
RNA
RNAi
RT-PCR
SEF
SGR
SH-EP
SMD
SNARE
ssVSS
SYP
TAIL-PC
TAIR
T-DNA
T-SNAR

TGN
TMD
tvs
UTR
VCL1
V-SNAR

VPE
VPS
VSP
VSR
VSS
VTH
VTI

NTPP	=N-Terminal Propeptide
NtSyr1	=Tobacco Syntaxin Related Protein
OSM1	=Osmotic Stress-Sensitive Mutant
PA	=Protease-Associated
PAC	=Precursor Accumulating Vesicle
PBS	=Phosphate Buffered Saline
PCR	=Polymerase Chain Reaction
PIN	=Pin-Formed
PPV	=Precursor Protein Vesicle
PrA	=Proteinase A
PrB	=Proteinase B
PSV	=Protein Storage Vacuole
PTGS	=Post-Transcriptional Gene Silencing
PV72	=Precursor Vesicle Protein of 72kDa
PVC	=Prevacuolar Compartment
RER	=Rough Endoplasmic Reticulum
RNA	=Ribonucleic Acid
RNAi	=RNA interference
RT-PCR	=Reverse Transcription-Polymerase Chain Reaction
SEF	=Soybean Embryo Factor
SGR	=Shoot Gravitropism
SH-EP	=Sulfhydrol Endopeptidase
SMD	=Stanford Microarray Database
SNARE	=Soluble N-Ethylmaleimide-Sensitive Factor Adaptor Protein Receptor
ssVSS	=Sequence-Specific Vacuolar Sorting Signal
SYP	=Syntaxin of Plants
TAIL-PCR	=Thermal Asymmetric Interlaced Polymerase Chain Reaction
TAIR	=The Arabidopsis Information Resource
T-DNA	=Transferred Deoxyribonucleic Acid
T-SNARE	=Target- Soluble N-Ethylmaleimide-Sensitive Factor Adaptor Protein Receptor
TGN	=*Trans*-Golgi Network
TMD	=Transmembrane Domain
tvs	=*trans-vacuolar strands*
UTR	=Untranslated Region
VCL1	=Vacuoleless1
V-SNARE	=Vesicle Soluble N-Ethylmaleimide-Sensitive Factor Adaptor Protein Receptor
VPE	=Vacuolar Processing Enzyme
VPS	=Vacuolar Protein Sorting
VSP	=Vegetative Storage Protein
VSR	=Vacuolar Sorting Receptor
VSS	=Vacuolar Sorting Signal
VTH	=VPS-Ten Homologue
VTI	=VPS-Ten Interacting Factor

Chapter 1

Introduction

An Overview of Protein Trafficking Through the Endomembrane System

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I. Introduction

The eukaryotic cell is compartmentalized into numerous types of organelles that perform specific functions. In order for organelles to perform their functions, proteins must be correctly targeted to the appropriate compartment. Protein targeting to organelles such as the nucleus, chloroplast, mitochondria, and other organelles occurs from the cytoplasm directly to the organelle. The endoplasmic reticulum (ER), Golgi, vacuole, and plasma membrane are connected through the endomembrane system (Figure 1.1). Soluble proteins are delivered to organelles of the endomembrane system via vesicle trafficking (Alberts et al., 1994)(Figures 1.2 and 1.3). In general, the process of vesicle trafficking from one organelle to another occurs in the following way: cargo proteins are brought to a specific domain of the originating organelle by aggregation, bulk flow, or through interaction with protein sorting receptors (Figure 1.2). Adaptor proteins on the surface of the developing vesicle recruit coat proteins to the budding vesicle. Coatamer is the coat protein that surrounds vesicles that emerge from the *cis*-Golgi and the ER whereas clathrin coats the vesicles that bud from the *trans*-Golgi Network (TGN) and the plasma membrane. The combined effects of the coat proteins and other accessory proteins force the vesicle to bleb away from the organelle (Figure 1.2). Then, the coat proteins are shed from the vesicle after its release from the originating organelle (Figure 1.3).

The shedding of the vesicle coat exposes SNAREs (soluble N-ethylmaleimide-sensitive factor adaptor protein receptor) on the vesicle surface that help specify the identity and appropriate destination organelle of that vesicle (Figure 1.3).

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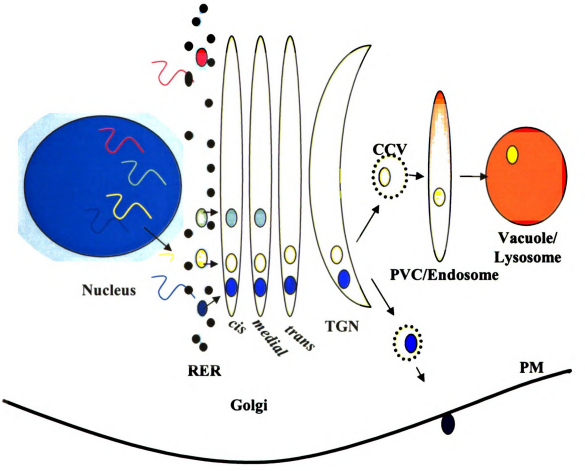
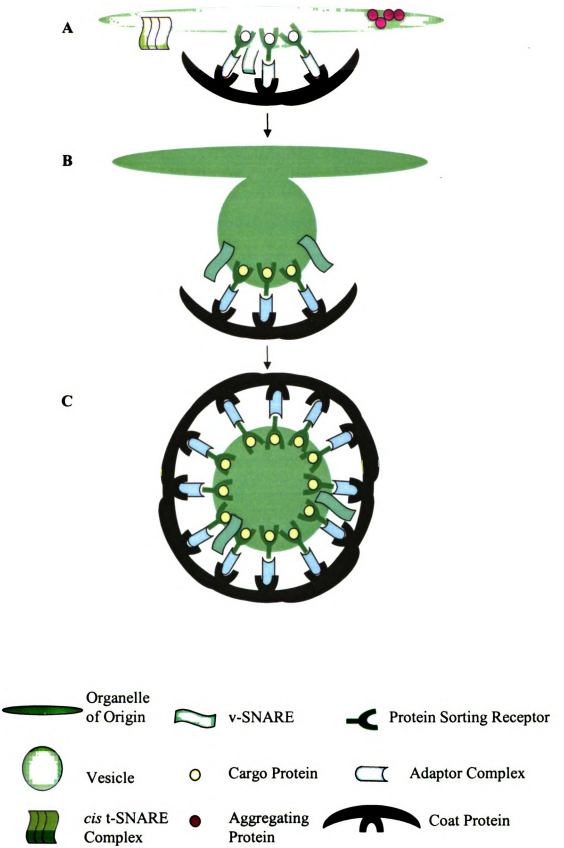


Figure 1.1. A schematic of the endomembrane system. mRNAs encoding soluble endomembrane proteins are exported from the nucleus and cotranslationally inserted into the rough endoplasmic reticulum (RER) (colored lines). ER resident proteins remain in the ER (red circles), whereas other proteins are delivered to the Golgi via vesicle trafficking (green, yellow, and blue circles). Golgi-resident proteins are delivered to the *cis, medial,* or *trans* Golgi (green circles). At the TGN, a sorting event occurs in which secreted proteins (dark blue circles) are delivered to the plasma membrane by the default pathway and vacuolar/endosomal proteins (yellow circles) are packaged into vesicles (usually CCV) and are delivered to the vacuole/lysosome via an endosome/PVC. Adapted from Alberts, et al., 1994.

Figure 1.2. Many factors are required for vesicle formation. The first step of vesicle formation is that the cargo proteins are recognized by protein sorting receptors or aggregate into a specific area of the organelle (A). v-SNARES also accumulate on the surface of the developing vesicle for later identification of the vesicle. The cytoplasmic tail of the receptor protein interacts with the coat forming complex to affix a coat around the budding vesicle (B). The coat protein and other factors pinch off the membrane to release the budding vesicle (C). Adapted from Alberts, 1994.


A
B
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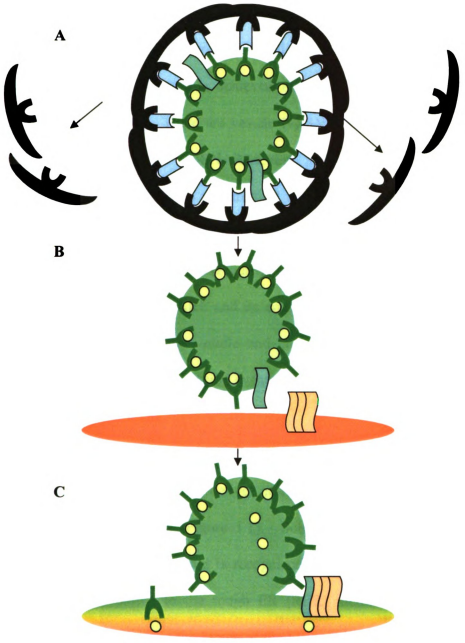
Organelle of Origin
v-SNARE
Protein Sorting Receptor
Vesicle
Cargo Protein
Adaptor Complex
cis t-SNARE Complex
Aggregating Protein
Coat Protein

Figure 1.3. The interaction of SNAREs drives membrane fusion between a vesicle and its target organelle. The coat protein is shed from the vesicle after budding from the originating organelle (A). The v-SNAREs are thus exposed on the surface of the vesicle and can interact with the t-SNAREs of the target organelle (B). Membrane fusion occurs and the contents of the vesicle are deposited into the destination organelle (C). At this step, the receptor protein releases the cargo in the destination organelle. Adapted from Alberts, 1994.


A
B
C
Cargo Protein
Protein Sorting Receptor
Vesicle
v-SNARE
Coat Protein
Adaptor Complex
Destination Organelle
cis t-SNARE Complex

Because vesicles are arriving and departing from multiple organelles, it is necessary for the vesicle and target organelle to be appropriately identified. Proper identification of compartments is achieved through the action of SNARE proteins (Sanderfoot et al., 2000). There are vesicle (v)-SNAREs that are localized to specific vesicles as well as target (t)-SNAREs that are localized to the membranes of compartments receiving vesicles (Figures 1.2 and 1.3). Three t-SNAREs on the target organelle form a *cis*-SNARE complex that can interact with a v-SNARE on the surface of a vesicle destined for the organelle (Figures 1.2 and1.3) (Surpin and Raikhel, 2004). The interaction between the v-SNARE and its cognate *cis*-SNARE complex allows for the membrane fusion between the vesicle and target organelle (Figure 1.3) (Surpin and Raikhel, 2004). The membrane fusion event allows the deposition of the vesicle contents into the target compartment.

The actions of vesicle budding and fusion occur at all points in the endomembrane system in order to deliver newly synthesized proteins to the appropriate organelle for their functions (Figure 1.1). Soluble endomembrane proteins have an amino-terminal signal peptide that is recognized by the ribosomes so that the nascent polypeptide is transported to the rough ER (RER) membrane. The protein is then cotranslationally inserted into the lumen of the ER and the signal peptide is cleaved from the protein. Most proteins that belong in downstream compartments of the endomembrane system are delivered to the *cis*-Golgi and onward to the TGN. At the TGN, a sorting event occurs in which proteins that have positive sorting information are separated away from secreted proteins. Proteins without any intracellular sorting information are secreted to the plasma membrane by the default pathway. Vacuolar

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proteins carry sorting signals that direct their delivery to vacuoles and will be discussed in more detail later (Vitale and Raikhel, 1999).

Plants have multiple types of vacuoles and, based on genome sequencing projects, encode many more genes than other organisms to carry out trafficking through the endomembrane system (Paris et al., 1996; Sanderfoot et al., 2000; Park et al., 2004). For example, one vacuolar sorting receptor, vacuolar protein sorting 10 (Vps10), has been characterized in *S. cerevisae* (Marcusson et al., 1994) and two other putative vacuolar sorting receptors have been identified in the *S. cerevisae* (Cooper and Stevens, 1996; Westphal et al., 1996). Likewise, two receptors that deliver proteins from the TGN to the lysosome have been characterized in humans (Dahms and Hancock, 2002). By sequence homology, *Arabidopsis* encodes seven genes that may function as vacuolar sorting receptors (VSR) (Hadlington and Denecke, 2000; Shimada et al., 2003). It is still not clear why higher plants encode so many more vacuolar sorting receptors than yeast or mammals. The focus of my research has been to understand the expression patterns and/or functions of a family of putative vacuolar sorting receptors in *Arabidopsis*. A background of vacuolar/lysosomal transport that has been characterized in other systems is useful to my study since many of the models and ideas about vacuolar transport in plants stem from what has been learned in animals and yeast.

II. Lysosomal Transport in Animal Cells

The lysosome is the animal cell equivalent of the plant cell lytic vacuole. The lysosomes are acidic compartments that degrade proteins. Protein trafficking to the lytic vacuole is essential. The absence of vacuolar trafficking in humans leads to I-Cell

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Disease, which kills patients during childhood (Dahms and Hancock, 2002). Proteins that are targeted to the lysosome, such as proteases, are modified in the Golgi with a mannose-6-phosphate (M-6-P) moiety that serves as a lysosomal sorting signal (von Figura and Hasilik, 1986).

Two mannose-6-phosphate receptors (MPR) have been isolated and characterized, the 46kDa cation-dependent MPR (CD-MPR) and the ~300kDa insulin-like growth factor II/MPR (IGFII/MPR) (Sahagian et al., 1981; Hoflack and Kornfeld, 1985a, b; Dahms and Hancock, 2002). MPRs are type I transmembrane proteins that contain an N-terminal signal peptide, a long extracytoplasmic domain and a short cytoplasmic tail (Dahms and Hancock, 2002). MPR localizes to the Golgi, clathrin-coated vesicles (CCV), plasma membrane, and endosomes, but not in lysosomes (Fischer et al., 1980; Sahagian et al., 1981; Campbell and Rome, 1983; Geuze et al., 1984; Klumperman et al., 1993; Le Borgne and Hoflack, 1997). Lysosomal proteins carrying the M-6-P signal also localize to the late Golgi, TGN, and CCVs (Campbell and Rome, 1983; Geuze et al., 1984; Geuze et al., 1985; von Figura and Hasilik, 1986). Therefore, receptor and cargo proteins are colocalized upstream of the lysosome. Furthermore, MPRs interact with the M-6-P moiety of lysosomal enzymes with high affinity and dissociate in pH conditions that resemble an intermediate endosomal compartment (Sahagian et al., 1981; Fischer et al., 1982; Tong et al., 1989; Tong and Kornfeld, 1989; Dahms and Hancock, 2002). The cytoplasmic tail of MPR has a tyrosine motif and other motifs that are responsible for its subcellular localization (Dell'Angelica and Payne, 2001). From these observations, a model of MPR function in lysosomal trafficking has developed (Dahms and Hancock, 2002). The M-6-P moiety of the cargo protein interacts with MPR in the in the neutral

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pH environment of the TGN. The cytoplasmic tail of MPR interacts with clathrin coat adaptor proteins and other factors to package the cargo and receptor into a CCV. The CCV buds from the TGN and is delivered to the endosome. MPR releases the cargo in the more acidic environment of the endosome. Finally, the cargo is delivered to the lysosome while MPR is recycled either to the TGN or to the plasma membrane.

III. Vacuolar Transport in *S. cerevisiae*

In yeast, as in animals, the primary function of the vacuole is protein degradation. However, the vacuole is not an essential organelle in yeast (Horazdovsky et al., 1995). The dispensability of the vacuole has made yeast a useful system for studying trafficking through the endomembrane system (Horazdovsky et al., 1995). There are multiple pathways to the vacuole in *S. cerevisiae* that include vesicle trafficking from the TGN, cytoplasm-to-vacuole traffic, endocytosis, as well as autophagy. Two of the vesicle-mediated pathways from the TGN to the vacuole are the carboxypeptidase Y (CPY) pathway which delivers proteins to the vacuole via a prevacuolar compartment and the alkaline phosphatase (ALP) pathway that bypasses the prevacuolar compartment (PVC) (Horazdovsky et al., 1995; Cowles et al., 1997; Piper et al., 1997).

Unlike animals, plant and fungal vacuolar proteins have peptide-based vacuolar sorting signals (VSSs) that are part of the translated protein, rather than a sugar modification to the protein. For example, mutations of single amino acids within the [Q]-[R]-[P]-[L] motif of CPY results in secretion of the protein (Valls et al., 1987; Valls et al., 1990). This motif is also sufficient to redirect normally secreted proteins to the

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vacuole (Johnson et al., 1987). Therefore, the QRPL motif of the CPY precursor is necessary and sufficient to direct a protein to the yeast vacuole.

In *S. cerevisiae*, a vacuolar sorting receptor was identified in a mutant screen to find lines that secrete CPY, rather than deliver it to the vacuole (Marcusson et al., 1994). Vps10p (vacuolar protein sorting 10) is a type I transmembrane protein with a large lumenal domain (1373 amino acids), a transmembrane domain of 20 amino acids, and a 160-amino acid cytoplasmic tail (Figure 1.4) (Horazdovsky et al., 1995; Jorgensen et al., 1999). The lumenal domain begins with an amino terminal signal peptide for insertion into the ER membrane. The remainder of the lumenal domain is composed of domain 1 and domain 2 (Figure 1.4) (Horazdovsky et al., 1995; Jorgensen et al., 1999). Each domain has a cysteine-rich region (Jorgensen et al., 1999). The cytosolic domain has a tyrosine motif (Cooper and Stevens, 1996). Thus, Vps10 is very similar in structure to MPR.

Biochemical and microscopy approaches indicate that both CPY and Vps10 localize to the late Golgi, CCVs, and a prevacuolar compartment (Vida et al., 1993; Marcusson et al., 1994; Cooper and Stevens, 1996; Seaman et al., 1997; Deloche et al., 2001). Furthermore, CPY interacts with Vps10 in the late Golgi (Marcusson et al., 1994; Cooper and Stevens, 1996). These data indicate that vacuolar proteins interact with the Vps10 receptor in the late Golgi and both proteins are delivered to the PVC via CCVs. This pathway is analogous to the MPR pathway of animals. The stoichiometry of the Vps10-CPY interaction is 1:1, however, CPY is expressed 20 times higher than the Vps10 (Cooper and Stevens, 1996). This suggests that the Vps10 receptor must be recycled for multiple rounds of vacuolar sorting (Cooper and Stevens, 1996).

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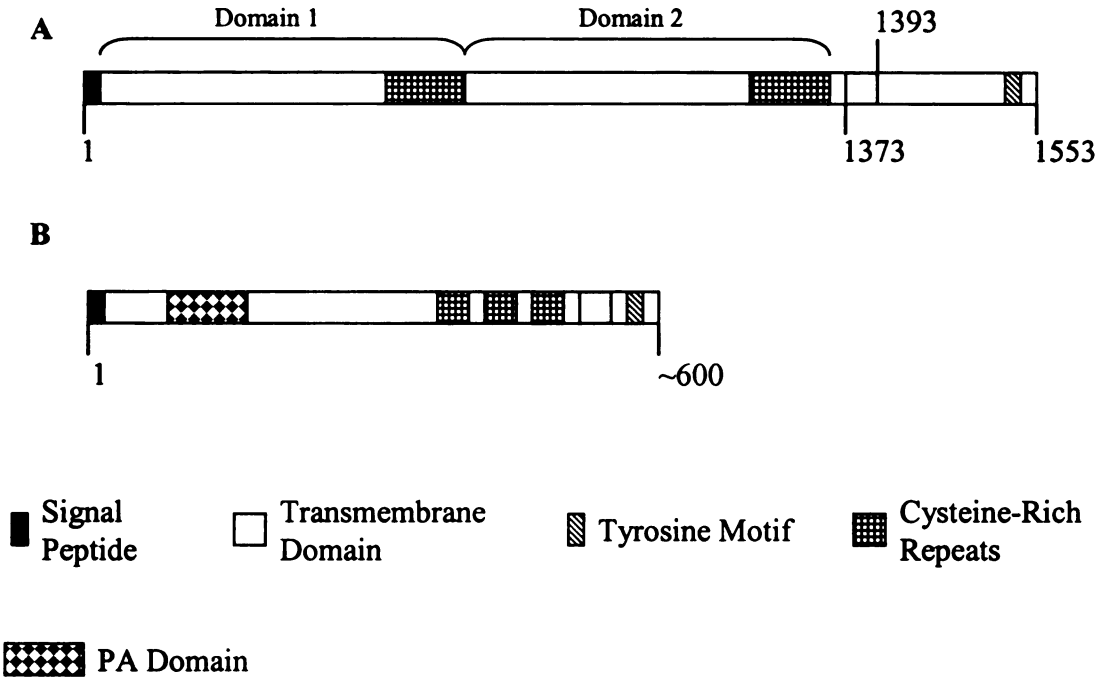

Figure 1.4. Structure of the *S. cerevisiae* Vps10p vacuolar sorting receptor and a plant vacuolar sorting receptor. A schematic representing the structure of Vps10p (A) (Cooper & Stevens, 1996; Westphal, et al., 1996; Jorgensen, et al., 1990) and a typical plant VSR (B) (Ahmed, et al., 1997; Shimada, et al., 2003; Paris, et al., 1997). The lumenal region of Vps10 is divided into domain 1 and domain 2 with each domain carrying a cysteine-rich motif. The cytoplasmic tail has a Tyrosine motif. Plant VSRs also have cysteine-rich repeats in the lumenal domain and a tyrosine motif in the c-terminal tail. They also have a PA domain in the lumenal region of the protein. The schematic is not drawn to scale.

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The C-terminal tail contains a tyrosine-based motif that is thought to be responsible for the recycling of Vps10p to the Golgi (Deloche et al., 2001). Mutation of the tyrosine in the signal leads to degradation of Vps10p in the vacuole and secretion of CPY (Deloche et al., 2001). Candidates for proteins that interact with the C-terminal tail of Vps10p to direct its subcellular localization include adaptor protein-1 (AP-1), sorting nexins such as Vps5p, Vps29p, Vps30p, and Vps35p (Horazdovsky et al., 1997; Seaman et al., 1997). Therefore, it is likely that Vps10p relies on these proteins to be recycled for further rounds of lysosomal trafficking.

From the accumulated data, a model of Vps10p function is proposed that is analogous to lysosomal sorting in animal cells (Horazdovsky et al., 1995). The QRPL motif of precursor CPY interacts with the second domain of Vps10p in the late Golgi (Marcusson et al., 1994; Cooper and Stevens, 1996). The receptor-CPY complex is packaged into CCVs and delivered to a PVC (Vida et al., 1993; Deloche et al., 2001). The CPY precursor dissociates from Vps10p in the PVC (Cooper and Stevens, 1996). CPY is then delivered to the vacuole and Vps10p is recycled to the Golgi (Horazdovsky et al., 1995; Horazdovsky et al., 1997).

Vps10p is not the receptor for all soluble vacuolar proteins, nor is the CCV pathway the only pathway for protein transport to the vacuole. For example, the vacuolar localization of the proteinase A (PrA), proteinase B (PrB), and ALP are not significantly affected in the Δ*vps10* mutant (Marcusson et al., 1994). Therefore, PrA and PrB must be delivered to the vacuole by a Vps10-independent pathway (Marcusson et al., 1994). The *S. cerevisiae* genome encodes two more genes, VTH1 and VTH2 (Vps Ten Homologue) that show significant similarity to VPS10 (Cooper and Stevens, 1996; Westphal et al.,

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1996). The proteins are 70% identical to Vps10 over the whole protein, specifically, there is 50% identity in the first domain and 88% identity in the second domain (Westphal et al., 1996). However, they are not expressed to detectable levels under the conditions studied (Westphal et al., 1996). So, it is not known whether these are functional receptors.

IV. Soluble Vacuolar Protein Transport in Plants

The plant cell vacuole is an essential organelle (Rojo et al., 2001). *Arabidopsis* embryos that cannot form a vacuole, such as the *vacuoless1* mutant accumulate autophagosome structures and die in the torpedo stage (Rojo et al., 2001). Transport through the endomembrane system is important to a plant's ability to respond to its environment (Surpin and Raikhel, 2004). For example, the *Arabidopsis* VTI (Vps10-interacting factor) SNAREs mediate the gravitropic and starvation responses (Surpin et al., 2003), whereas NtSyr1 mediates stress response pathways in tobacco (Leyman et al., 2000). Other components of vesicle trafficking in plants also play important roles in signal transduction, and solute homeostasis (Weintraub, 1952; MacRobbie, 1999; Kato et al., 2002; Zhu et al., 2002). Plants encode more genes whose proteins function in the endomembrane system than other organisms and direct proteins to multiple types of vacuoles (Figure 1.5) (Paris et al., 1996; Sanderfoot et al., 2000; Park et al., 2004).

The lytic vacuole is an acidic organelle equivalent to the yeast vacuole and animal lysosome. The lytic vacuole is responsible for ion homeostasis, storage of secondary metabolites such as anthocyanins, as well as protein turnover (Surpin and Raikhel, 2004).

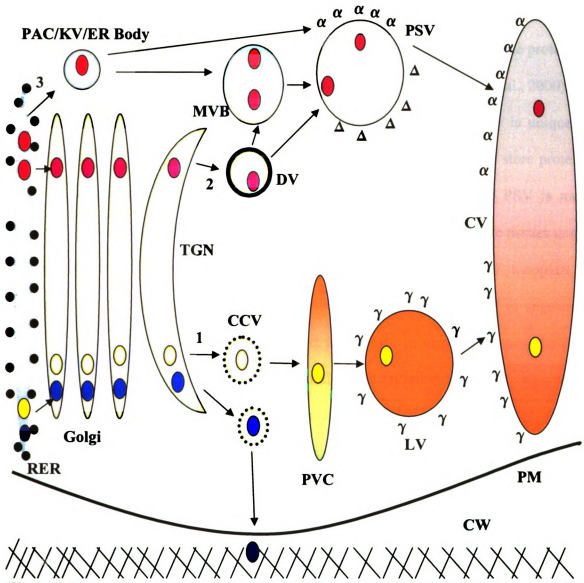

Figure 1.5. Protein transport through the endomembrane system of plants. Proteins are cotranslationally inserted into the rough endoplasmic reticulum (RER). Some proteins (red circles) aggregate into precursor accumulating vesicles (PAC), KDEL vesicles (KV), or ER bodies that bleb off the ER and either fuse with multivesicular bodies (MVB) or are delivered to the protein storage vacuole (PSV), marked by αTIP in developing seeds or by ΔTIP in vegetative cells (3). Other proteins are delivered to the Golgi via vesicle trafficking (red, blue, and yellow circles). Storage proteins (red circles) are packaged into dense vesicles that can fuse with MVBs or be delivered directly to the PSV (2). Soluble proteins intended for the lytic vacuole (yellow circles) are packaged into clathrin coated vesicles (CCV) and delivered to the lytic vacuole (LV), marked by γTIP and sometimes αTIP, via the prevacuolar compartment (PVC). Secreted proteins (blue circles) are also packaged into CCVs that are delivered to the plasma membrane (PM) and cell wall (CW). The separate pathways to the vacuole are maintained even in cell types in which the PSV and LV fuse to form the large central vacuole (CV).

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The tonoplast of the lytic vacuole is marked by the γ-TIP aquaporin (Figure 1.5) (Paris et al., 1996; Jauh et al., 1999). Proteases are abundant in the lytic vacuole. Some proteases, such as Aleurain, are now common markers for the lytic vacuole (Ahmed et al., 2000).

The protein storage vacuole (PSV) is a neutral pH organelle that is unique to plants (Figure 1.5) (Vitale and Raikhel, 1999). The role of the PSV is to store proteins such as lectins, chitinases, legumins (Vitale and Raikhel, 1999). The PSV is most commonly associated with developing seeds but can also form in vegetative tissues under specific conditions (Paris et al., 1996; Jauh et al., 1999). The PSV tonoplast is characterized by the presence of α-TIP in seeds and with α-TIP or Δ-TIP in vegetative tissues (Figure 1.5) (Paris et al., 1996; Jauh et al., 1999). In many cell types, the lytic vacuole and the PSV fuse to form a large, central vacuole (Paris et al., 1996). As a result of the fusion, the central vacuole tonoplast contains α-TIP and γ-TIP (Figure 1.5) (Vitale and Raikhel, 1999). The central vacuole contains both storage and lytic vacuolar soluble proteins.

Positive sorting information is required for protein sorting to the vacuole and, like *S. cerevisae*, plants use peptide based VSSs that are part of the translated protein. Three types of vacuolar sorting signals have been identified in plants (Figure 1.6) (Vitale and Raikhel, 1999). They include the N-terminal propeptide (NTPP; Table 1.1), the C-terminal propeptide (CTPP; Table 1.2), and an internal signal. It should also be mentioned that the C-terminal ER retention signal, KDEL, can also function as a VSS under certain conditions (Vitale and Raikhel, 1999; Schmid et al., 2001; Okamoto et al., 2003).

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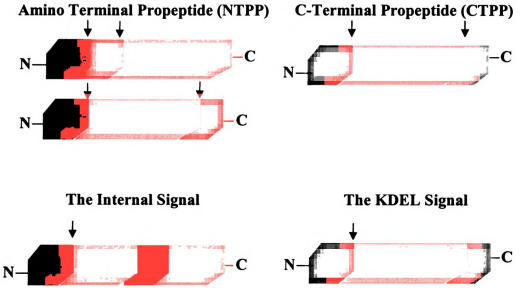

Figure 1.6. Vacuole sorting signals in plants. The schematic depicts three types of vacuolar sorting signals (VSS) that have been identified in plants and the KDEL signal. All of the proteins have a signal peptide (blue box) for cotranslational insertion in into the ER. The signal peptide is cleaved from the protein after insertion into the ER (arrow after blue box). The NTPP (red box) is a sequence specific VSS. The signal is cleaved from the mature protein after delivery to the vacuole (arrow after red box). The CTPP (grey box) is a C-terminal VSS that is not sequence specific, but may form a distinctive tertiary structure. The CTPP is also cleaved from the mature protein after delivery to the vacuole (arrow before grey box). The internal signal (green box) is necessary for delivery to the vacuole and is part of the mature protein. The KDEL signal (purple box) normally functions as an ER retention signal, however, can act as a vacuolar sorting signal under specific conditions. (Reviewed in Vitale & Raikhel, 1999).

The NTPP and CTPP signals are amino acid sequences that are cleaved from the mature protein after delivery to the vacuole (Figure 1.6). However, each type of VSS is thought to direct proteins to different vacuoles by different pathways.

Table 1.1. N-Terminal Propeptides

Protein	Amino Acid Sequence of Signal (amino acid position)	Reference
N-terminal NTPP		
Sweet Potato Sporamin	(22)HSRFNPIRLPTTHEPA...	(Matsuoka and Nakamura, 1991)
Barley Aleurain	(22)SSSFADSNPIRPVTDRAAS...	(Holwerda et al., 1992)
Arabidopsis Aleurain	(22)ANIGFDESNPIRMVSDGLR...	(Ahmed et al., 2000)
Potato PT20	(19)STFTSKNPINLPSDA...	(Koide et al., 1999)
Internal NTPP		
Castor Bean Ricin	(303)SLLIRPVVPNFN...(576)	(Frigerio et al., 2001)
C-terminal NTPP		
Brazil Nut 2S Albumin	(130)NLPSMRCPMGGSIAGF(C-terminus)	(Kirsch et al., 1996)
Arabidopsis 2S Albumin	(150)VCPNIPSFPS(C-terminus)	(D'Hondt et al., 1993)

Table 1.2. C-Terminal Propeptides

Protein	Amino Acid Sequence of Signal	Reference
Barley Lectin	VFAEAIAANSTLVAE	(Bednarek et al., 1990)
Tobacco Chitinase	GLLVDTM	(Neuhaus et al., 1991)
Phaeseolin	AFVY	(Frigerio et al., 1998; Holkeri and Vitale, 2001)
α'-Soybean β-Conglycinin	PLSSILRAFY	(Nishizawa et al., 2003)
Tobacco Proteinase Inhibitor	SEYASKVDEYVGENDLQKSKVAVS	(Miller et al., 1999)
Tobacco AP24	QAHPNFPLEMPGSDEVAK	(Melchers et al., 1993)
Horseradish Peroxidase C1a	LLHDMVEVVDFVSSM	(Matsui et al., 2003)
B-1,3-Glucanase	VSGGVWDSSVETNATASLVSEM	(Melchers et al., 1993)

Just as multiple pathways to the vacuole exist in other organisms, plants are thought to deliver proteins to the vacuole by a dense vesicle pathway (Figure 1.5, pathway 2), a precursor protein vesicle (PPV) pathway (Figure 1.5, pathway 3), and a CCV pathway (Figure 1.5, pathway 1) (Hayashi et al., 2001; Schmid et al., 2001;

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Okamoto et al., 2003; Rojo et al., 2003a). It is likely that there is cross-talk between these pathways (Surpin and Raikhel, 2004).

The evidence for the presence of multiple pathways to vacuoles in plants first came from the observation that the trafficking of NTPP and CTPP proteins have different sensitivities to wortmannin, a PI-3-kinase inhibitor (Matsuoka et al., 1995). PSV proteins localize to dense vesicles and not to CCVs (Hohl et al., 1996). Therefore, soluble PSV proteins are packaged into dense vesicles at the TGN and are delivered to the protein storage vacuole (Hohl et al., 1996; Paris et al., 1996). Proteins with CTPP signals are thought to travel by the dense vesicle pathway (Matsuoka et al., 1995; Hohl et al., 1996). While there is no conserved sequence motif, characterized CTPPs tend to be hydrophobic and are strictly located at the C-terminus of a protein (Bednarek et al., 1990; Dombrowski et al., 1993; Neuhaus et al., 1994). A receptor for CTPP proteins has not been identified. Some researchers have speculated that aggregation of these proteins in the Golgi is the mechanism of their trafficking from the TGN (Vitale and Raikhel, 1999). This model is analogous to a regulated secretion model that has been described in animals (Vitale and Denecke, 1999). However, the vacuolar sorting of CTPP proteins is saturable, suggesting that a receptor is involved in the process (Neuhaus et al., 1994).

Some proteins that reside in the storage vacuole actually aggregate in the ER into vesicles that bleb from the ER (Okamoto et al., 1994; Hayashi et al., 2001; Schmid et al., 2001; Okamoto et al., 2003; Rojo et al., 2003b). These vesicles are called ER bodies, precursor accumulating vesicles (PAC), PPVs, and KDEL vesicles (KV) (Shimada et al., 1997; Hayashi et al., 2001; Tsuru-Furuno et al., 2001; Rojo et al., 2003b). The PPVs can fuse with dense vesicles to form multivesicular bodies (MVBs) which are delivered to the

PSV (Tse et al., 2004). It has even been suggested that MVBs may be a PVC for the PSV (Tse et al., 2004). Proteins that have KDEL signals and/or novel, uncharacterized signals use this pathway. The KDEL signal normally functions as an ER-retention signal when present at the C-termini of proteins. However, some proteins that have C-terminal KDEL signals, such as the papain type cysteine proteinase, SH-EP, are delivered to the vacuole during senescence and programmed cell death via ER-derived vesicles (Vitale and Denecke, 1999; Tsuru-Furuno et al., 2001). What is still unclear is how the KDEL signal can function as an ER retention signal and as a VSS. The context of the protein and the environmental conditions likely regulate this process although the mechanisms of such regulation are still unclear. The KDEL signal can confer ER and vacuolar localization to green fluorescent protein (GFP) when KDEL is fused to the C-terminus of GFP and stably expressed from a constitutive promoter in *Arabidopsis* (Di Sansebastiano et al., 1998; Hayashi et al., 2001). Further insight into the dual functions of the KDEL signal may come from high-throughput proteomics and protein localization projects.

Proteins that have NTPP VSSs are thought to be delivered to the lytic vacuole by a CCV pathway that is analogous to what has been described in yeast and animals (Ahmed et al., 2000). The NTPP has been extensively characterized in plants and has a conserved sequence motif. Site-directed mutagenesis of the prosporamin propeptide and subsequent transient expression in Tobacco BY-2 cells revealed that the consensus NTPP motif is: [preferably Asn]-[not acidic]-[Ile or Leu]-[X]-[Large and hydrophobic] (Matsuoka and Nakamura, 1999). Although the NTPP of known vacuolar proteins, such as Aleurain, is at the amino terminus, the placement of a functional NPIR motif is not restricted to the amino-terminus (Kirsch et al., 1996; Koide et al., 1997; Frigerio et al.,

2001). An interesting example of this comes from studies of the ricin precursor (Frigerio et al., 2001). The ricin precursor was previously thought to have an internal sorting signal (Frigerio et al., 2001). However, it is now clear that the internal signal of ricin has an NPIR motif that directs ricin to the vacuole (Frigerio et al., 2001). Similar results have been observed for 2S albumin which also has an NPIR motif in its C-terminus (Kirsch et al., 1996; Shimada et al., 2002). What is curious about these precursor proteins is that these are proteins that are localized to the PSV, not to the lytic vacuole.

Vacuolar Sorting Receptors of Plants

Receptors for NTPP-containing proteins include pea binding protein-80 (BP-80), pumpkin PV72 (precursor vesicle protein of 72kDa), and *Arabidopsis* AtVSR1/AtELP (*Arabidopsis* vacuolar sorting receptor 1/*Arabidopsis* epidermal growth factor receptor-like protein) (Kirsch et al., 1994; Ahmed et al., 1997; Shimada et al., 2002). The pea BP-80 was identified by affinity chromatography of CCVs to identify proteins which bind to the NTPP of Barley Aleurain (Kirsch et al., 1994). The pumpkin PV72 was isolated from precursor accumulating (PAC) vesicles of developing seeds (Shimada et al., 1997). The *Arabidopsis* AtVSR1/AtELP was identified by a bioinformatics approach to identify cysteine-rich repeats that are present in receptor proteins of other systems (Ahmed et al., 1997).

Like MPR and Vps10, plant VSRs are type I transmembrane proteins with long lumenal domains followed by short transmembrane domains and short cytoplasmic domains (Figure 1.4) (Ahmed et al., 1997). The amino terminus of the plant VSR has a signal peptide for cotranslational insertion into the ER membrane (Ahmed et al., 1997).

A protease-associated (PA) domain follows the signal peptide (Mahon and Bateman, 2000). There are also three cysteine-rich epidermal growth factor (EGF) repeats within the lumenal domain, one of which is a calcium-dependent EGF repeat (Watanabe et al., 2002). The cytoplasmic domain has a tyrosine motif that interacts with the clathrin coat adaptor proteins at the TGN (Sanderfoot et al., 1998; Happel et al., 2004). It also contains a diacidic motif for export from the ER (Matsuoka and Bednarek, 1998).

AtVSR1 and BP-80 have been localized to the TGN, CCVs, the PVC, and colocalize with proteins destined for the lytic vacuole (Kirsch et al., 1994; Ahmed et al., 1997; Sanderfoot et al., 1998; Ahmed et al., 2000; Li et al., 2002). These locations are consistent with what is known about the vacuolar/lysosomal sorting receptors of animals and yeast, MPR and Vps10. On the other hand, PV72 localizes with PSV proteins in PAC vesicles (Shimada et al., 1997). BP-80 and AtVSR1 interact with NTPP signals *in* vitro in a sequence-specific and pH-dependent manner (Kirsch et al., 1994; Ahmed et al., 1997; Paris et al., 1997; Ahmed et al., 2000). These interactions are also similar to the CCV pathways of animals and yeast. However, PV72 interacts with 2S Albumin in a calcium-dependent manner (Shimada et al., 2002; Watanabe et al., 2002). Fluorescence-based ligand binding assays of deletion mutants of BP-80 indicate that while the region responsible for binding the ligand is N-terminal to the EGF repeats, the EGF repeats enhance the stability of the interaction (Cao et al., 2000). Similarly, PV72 interacts with 2S Albumin in a region outside of the EGF repeats in a calcium-dependent manner (Shimada et al., 2002; Watanabe et al., 2002). The third EGF repeat mediates the calcium-dependency of the interaction (Shimada et al., 2002; Watanabe et al., 2002). Thus, despite having different localization patterns and ligand dissociation requirements,

the mechanisms and domains required for the interaction are quite similar. BP-80 and PV72 both interact with cargo proteins in a region that is amino terminal to the EGF repeats and use the EGF repeats to stabilize the interaction between the VSR and the cargo protein. It is possible that the PA domain is the site of cargo protein interaction. The PA domain is a region upstream of the EGF repeats. The PA domain has been identified in diverse proteins (Mahon and Bateman, 2000). While the function of the PA domain is not known, it has been proposed that the PA domain is a site of ligand interactions (Mahon and Bateman, 2000).

The cytoplasmic tail of the plant VSR has a tyrosine motif like Vps10 and MPR (Sanderfoot et al., 1998; Bonifacino and Dell'Angelica, 1999; Shimada et al., 2002). The tyrosine motif is a short amino acid sequence composed of: [Y]-[X]-[X]-[Bulky hydrophobic] (Bonifacino and Dell'Angelica, 1999). The Tyrosine motif interacts with clathrin coat adaptor proteins either at the plasma membrane or at the TGN (Sanderfoot et al., 1998; Bonifacino and Dell'Angelica, 1999; Happel et al., 2004). The tyrosine motif of AtVSR1 and BP-80 specifically interacts with the TGN-specific adaptor proteins and does not interact with the plasma membrane specific adaptor protein (Sanderfoot et al., 1998; Happel et al., 2004). These results suggest that the cytoplasmic tail functions similarly to the C-terminal tail of Vps10 and MPR.

The current model of how this protein functions in the context of the CCV pathway is that the receptor interacts with an NTPP-containing protein at the TGN and both proteins are packaged into CCVs that are delivered to the PVC (Figure 1.7) (Ahmed et al., 2000). The receptor releases the cargo in the more acidic pH environment of the PVC and is presumably recycled to the TGN by vesicle trafficking whereas the cargo is

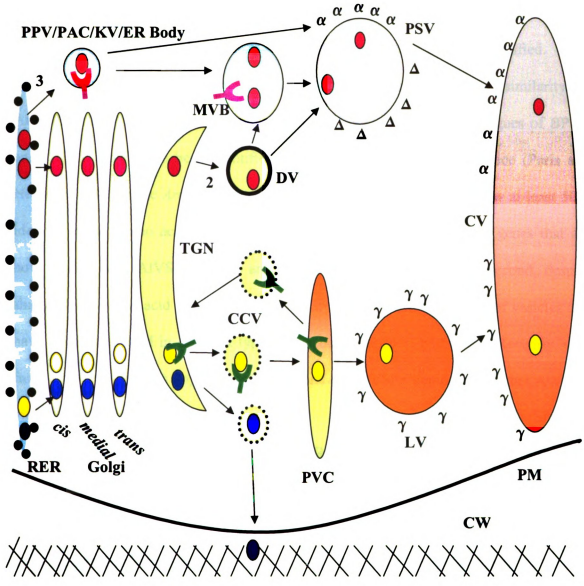

Figure 1.7. The current model of plant VSR function and the localization of other VSRs within the endomembrane system. Soluble proteins intended for the lytic vacuole (yellow circles) are recognized by plant VSRs (green hooks) and both are packaged into clathrin coated vesicles (CCV) and delivered to the PVC. At the PVC, the VSR dissociates from the cargo protein. The cargo protein is delivered to the lytic vacuole, whereas the VSR is recycled back to the TGN. VSRs have also been detected in PPVs as well as in MVBs (purple hooks) (Shimada, et al., 1997; Tsuru-Furuno, et al., 2001).

delivered to the lytic vacuole by either vesicle trafficking or fusion of the PVC with the vacuole. However, two observations indicate that this model is over-simplified. First, the *Arabidopsis* genome encodes multiple genes that show high similarity to AtVSR1/AtELP and thus may also function as VSRs. Multiple homologues of BP-80 and AtELP have since been identified in bean, pumpkin, wheat, and rice (Paris and Neuhaus, 2002). The *Arabidopsis* genome encodes seven genes that show at least 50% identity at the amino acid level to AtVSR1/AtELP. Rice encodes nine genes that are homologous with AtVSR1/AtELP and BP-80 (unpublished results). Second, despite sharing high amino acid identity, plant VSRs are found in different types of vesicles and have been implicated in different types of vacuolar transport pathways. Recently, data from a reverse genetics approach to understand plant VSRs demonstrated that AtVSR1 mediates the delivery of 2S Albumin to PSVs in developing *Arabidopsis* seeds (Shimada et al., 2003). These results indicate that AtVSR1 may also function like PV72 (Shimada et al., 2003). Furthermore, an AtVSR1 homolog was isolated from KV vesicles from developing seeds of *Vigna mun* (Tsuru-Furuno et al., 2001). While cross-talk between the vacuolar trafficking pathways may account for some of these results, it is now probable that the same family of receptors mediates vacuolar protein traffic by different pathways.

V. Thesis Overview

As mentioned above, the *Arabidopsis* family of VSRs contains seven members. These proteins have been called by different names in the past (Table 1.3). The current

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Table 1.3. Nomenclature for *Arabidopsis* Vacuolar Sorting Receptors

AGI Accession	Nomenclature I Previously Used	Nomenclature From Shimada et al., 2003
At3g52850	AtELP	AtELP/AtVSR1
At2g30290	AtVSR3	AtVSR2
At2g14740	AtVSR1	AtVSR3
At2g14720	AtVSR2	AtVSR4
At2g34940	AtVSR4	AtVSR5
At1g30900	AtVSR6	AtVSR6
At4g20110	AtVSR5	AtVSR7

For my dissertation, I have adopted the nomenclature from Shimada, et al., 2003. An important goal in this field is to identify the individual and overlapping functions of the AtVSRs. A keen understanding of this issue will help us understand why plant VSRs occur in such large families. My goals for this study were to determine the function(s) of these genes. One possibility is that each gene performs a similar function and the expression of each is restricted to specific cell types. The second possibility is that each protein performs a distinct function and thus there are multiple VSRs expressed in most plant cells. The third possibility is that the VSRs have completely redundant functions. It is also possible that a combination of these scenarios exists.

To address this question, I determined the expression patterns of the individual AtVSR genes and took advantage of the many bioinformatics tools available. To address the function of each gene at the protein level, I attempted to isolate knockouts of each gene, post-transcriptionally silenced individual members as well as the entire family of AtVSRs, and used biochemical means to identify the receptor of specific soluble vacuolar proteins. These approaches could lead to a greater understanding of the expression

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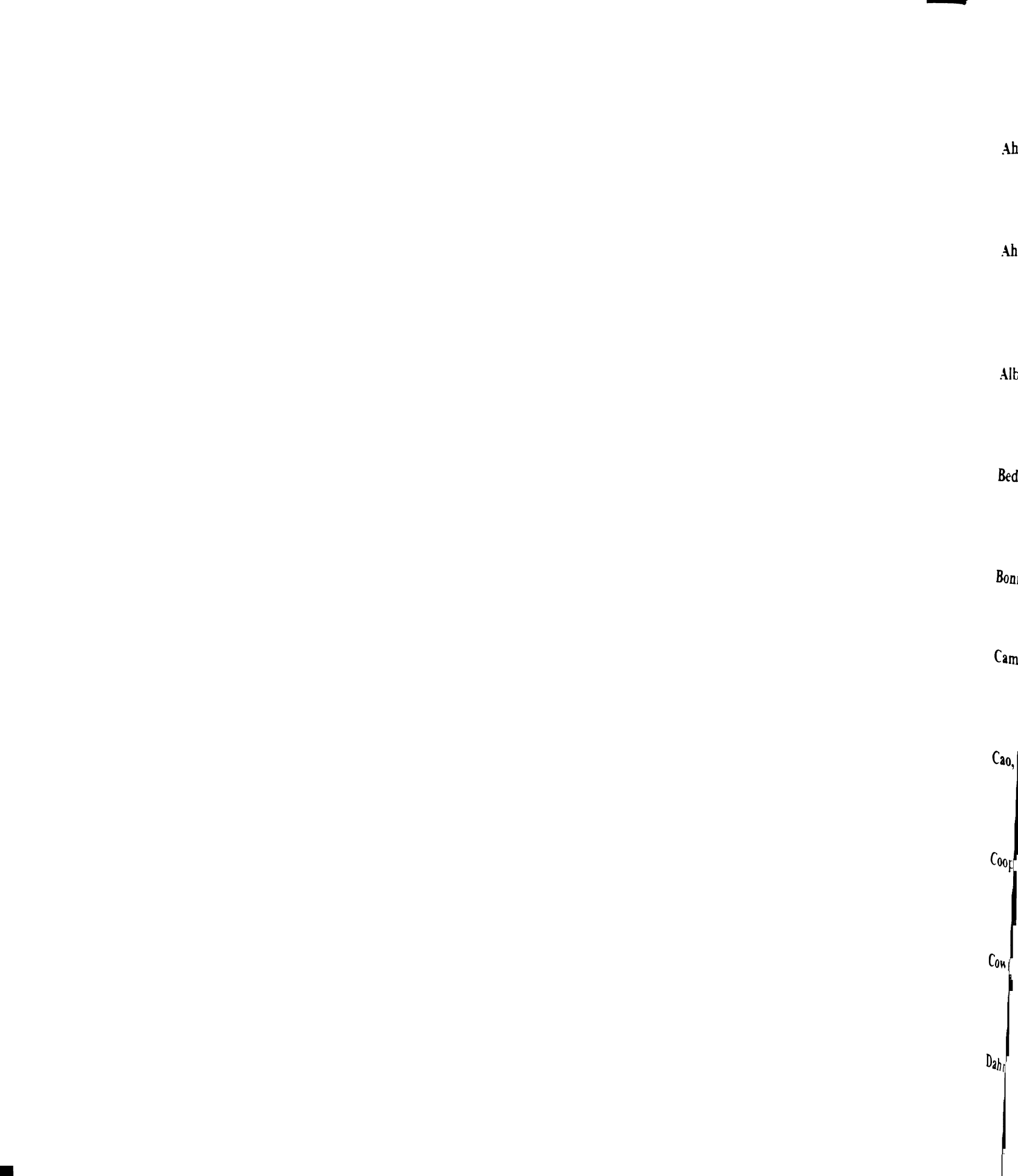
patterns of members of the VSR family as well as an understanding of the physiological function of at least one of the VSR proteins.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Chapter 2

Expression Analysis of the Vacuolar Sorting Receptor Family in *Arabidopsis*

Abstract

In plant cells, soluble proteins that are delivered to the vacuole via the endomembrane system contain a vacuolar sorting signal (VSS). Three types of VSSs have been identified in plants. One class of VSS is the N-terminal propeptide (NTPP) that is cleaved from the mature protein and contains a conserved peptide motif required for vacuolar sorting. A putative vacuolar sorting receptor (VSR) for NTPP-containing proteins is the *Arabidopsis* VSR1, formerly known as AtELP. AtVSR1 is a type I transmembrane protein with three cysteine-rich EGF repeats. Homologues of AtVSR1 have been identified in numerous plant species. The current model of VSR function is that the VSR interacts with NTPP-containing proteins at the *trans*-Golgi Network (TGN) and recruits them into clathrin-coated vesicles that are delivered to the prevacuolar compartment (PVC). At the PVC, the VSR releases the cargo and is returned to the TGN while the cargo proteins are sent to the vacuole. Phylogenetic analysis of all known plant VSRs indicated that the VSRs are subdivided into three groups. *AtVSR1* has six homologues in the *Arabidopsis* genome with at least one homolog in each of the three groups. Expression of all members of this gene family has been detected in various plant tissues by RT-PCR. The cell type-specific expression pattern of each gene was determined by promoter::GUS fusion and indicated that the AtVSRs have cell-type specific expression patterns. The overall results indicated that the seven members of the AtVSR gene family are not completely redundant.

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Introduction

The plant cell vacuole is a multifunctional organelle that is essential to plant growth and development (Rojo et al., 2001; Surpin and Raikhel, 2004). However, the identity and function of the vacuole is dependent on the proper targeting of vacuolar proteins. Soluble proteins are delivered to the vacuole via vesicle trafficking of the endomembrane system. These proteins are first cotranslationally inserted into the rough endoplasmic reticulum (RER). In the ER, some vacuolar proteins will segregate into ER bodies that bleb off the ER membrane and are delivered to the vacuole (Shimada et al., 1997; Tsuru-Furuno et al., 2001; Rojo et al., 2003). Most vacuolar proteins will be delivered to the Golgi. Proteins destined for the vacuole are sorted from secreted proteins at the TGN (Vitale and Raikhel, 1999). Vacuolar proteins are distinguished from secreted proteins by amino-acid sequence-based sorting signals.

Three types of vacuolar sorting signals (VSSs) have been identified in plants. All three signals are peptide sequences present in the protein. The amino-terminal propeptides (NTPP) and carboxy-terminal propeptides (CTPP) are amino acid sequences present at either end of the protein. Both signals are cleaved from the mature protein. There are also internal signals that are necessary for vacuolar delivery, but these are poorly characterized and not removed from the mature protein (Vitale and Raikhel, 1999). The type of vacuolar sorting signal appears to determine the pathway by which the protein is delivered to the vacuole (Matsuoka et al., 1995). Proteins that have CTPPs are packaged into dense vesicles and are delivered to protein storage vacuoles (PSVs). A CTPP receptor has not yet been identified. On the other hand, proteins that have an

NTPP are recognized by a vacuolar sorting receptor (VSR) in a sequence specific manner and are delivered to the lytic vacuole via the PVC (Surpin and Raikhel, 2004).

VSRs have been identified in numerous plants by many methods. The VSR from pea, BP-80 (binding protein of 80kDa), was isolated from pea clathrin-coated vesicles (CCVs) by its affinity for the NTPP of the soluble vacuolar protein, barley aleurain (Kirsch et al., 1994). The first VSR characterized from *Arabidopsis*, AtELP/AtVSR1 (Arabidopsis EGF receptor-like protein/Arabidopsis vacuolar sorting receptor 1), was identified by a bioinformatics approach based on the observation that receptor proteins in other systems have several conserved motifs (Ahmed et al., 1997). The VSR from pumpkin was identified by a protein analysis of precursor accumulating (PAC) vesicles (Shimada et al., 2002). Likewise, a VSR from *Vigna mungo* was identified from a protein analysis of KDEL-vesicles that travel from the ER to the vacuole (Tsuru-Furuno et al., 2001).

All of the plant VSRs share at least 50% sequence identity throughout the whole protein. The VSRs are type-1 transmembrane proteins that have a large lumenal domain and a short cytoplasmic domain (Ahmed et al., 1997). The lumenal domain has a signal peptide for insertion into the ER, followed by a protease-associated (PA) domain (Mahon and Bateman, 2000). Researchers have speculated that the PA domain may be the site at which the VSR interacts with protein cargo in the TGN (Cao et al., 2000; Mahon and Bateman, 2000; Watanabe et al., 2002). The lumenal domain also contains three epidermal growth factor (EGF) repeats that may regulate the protein-protein interactions with ligands (Ahmed et al., 1997; Watanabe et al., 2002). The cytoplasmic tail mediates

the interactions of the VSR with vesicle-forming machinery and contains sequences that pertain to the sorting of the VSR itself (Sanderfoot et al., 1998; Happel et al., 2004).

The model of VSR function in plants is that the VSR interacts with a soluble vacuolar protein in the near neutral pH of the TGN. Both proteins are packaged into CCVs and are delivered to the PVC. Then, the VSR releases the cargo into the more acidic environment of the PVC and is recycled back to the TGN while the cargo is sent to the vacuole (Ahmed et al., 2000). With the identification of VSRs in vesicles other than CCVs (Shimada et al., 1997; Tsuru-Furuno et al., 2001), and the observation that the *Arabidopsis* genome encodes seven putative VSRs (Hadlington and Denecke, 2000; Shimada et al., 2003), it is now clear that this model of VSR function is not necessarily wrong, but should be expanded. In order to develop a more comprehensive model of VSR function in plants, I addressed the functions of the individual *AtVSR* genes by analyzing the phylogenetic relationship between all the identified plant VSRs, and by determining the expression pattern for each member of the *Arabidopsis* gene family through multiple experimental approaches. Towards this end, I determined the expression patterns of each gene by RT-PCR and promoter::GUS fusions. I also used bioinformatics to learn more about the VSR expression patterns and to identify regulatory elements within each promoter.

Materials and Methods

Images in this dissertation are presented in color.

RT-PCR of the AtVSR Genes

Gene-specific primers were designed for each gene and synthesized by the MSU Macromolecular Structural Facility or Fisher Scientific: *ELP/VSR1* Forward = CTT GGG CTT TTC ACT CTC TCG TTT C; *ELP/VSR1* Reverse = TCC AAC TTT GCC TGA ACC TAT GC; *VSR2* Forward = TCC CAA TAC TCA ACT TTC TTC TCA AC; *VSR2* Reverse = GTT TTC ACT ATT TGG TTG TGT GTA C; *VSR3* Forward = CCT TGT CCT TCG AAT TTG TTC TTT G; *VSR3* Reverse = TCT AGA GTC CTT CCC GGG GAA TAA ATA GAT G; *VSR4* Forward = CTA TTG ACT AGC TTG TCC AGT TCT CCG TA; *VSR4* Reverse = AGT GCA AAG AGA AGA CAT GTC AGT GC; *VSR5* Forward = ATG GCT CGT GTG GGG TTG TAT TTG ACT ACC; *VSR5* Reverse; AGT CTT GGT TAA TGC TTT GGC TAT C; *VSR6* Forward = ATG TCT TTG ATT CAT AAA GGA GCC AC; *VSR6* Reverse = CAG AAG TTA ATC TCA GCT GTT GGT G; *VSR7* Forward = GAG ATG GGT TTA GTC AAC GGG AGA G; *VSR7* Reverse = GTA AAA GGC TCG GCT TCT GAT GGA AC. Total RNA was extracted from the following *Arabidopsis* tissues with the RNeasy Kit (Qiagen): dry seeds, seven-day old seedlings, three-weekold roots, young rosette leaves, mature rosette leaves, bolts, flowers, and green siliques. RT-PCR was carried out for each gene using the One-Step RT-PCR Kit (Qiagen) with 500 ng total RNA template and 20-25 cycles of RT-PCR, unless otherwise indicated. The PCR products were electrophoresed on a 1% agarose gel with ethidium bromide and photographed by a gel documentation system (BioRad). The DNA of all PCR products was sequenced by the University of California, Riverside (UCR) Center for Genomics DNA Sequencing Facility to confirm their identities.

Preparation of Constructs: Promoter::GFP:GUS Fusion Constructs

All PCR products used to generate the promoter fusion constructs were amplified from wild type Columbia genomic DNA using Takara Ex-Taq DNA Polymerase and following the manufacturer's protocol. The PCR products were purified using a PCR purification kit (Qiagen) and cloned into pGEM-T Easy (Promega). DNA sequencing of the PCR products and the constructs was done by the UCR DNA Sequencing Facility. After each construct was prepared as described below, they were transformed into wild type *Arabidopsis* (Columbia ecotype) by *Agrobacterium*-mediated stable transformation. Seeds collected from the transformed plants were germinated on agar containing Murashige Minimal Organics Medium, 30 mg/L hygromycin (Sigma), and 25mg/L Carbenecillin (Sigma).

35S::GFP:GUS

We received the pRJG23 construct as a generous gift from Dr. Jen Sheen. For ease of cloning into *pCAMBIA*, the *GFP::GUS* fragment of *pRJG23* was cloned into *pBS SK+* with an EcoRI/HindIII double digest. The *GFP::GUS* fragment was then cloned into *pCAMBIA 1300MCS* with a KpnI/SacI double digest and ligation to make *pCAMBIA::GFP:GUS.*

VSR1promoter::GFP:GUS

The 2520 bp region upstream of the *AtVSR1* start codon was amplified with the following primers: forward = AAG GTA AGA AAT ATT CTC TCA TTT TC; reverse = GAT ATC GAA ACG AGA GAG TGA AAA GAA GAA G. An EcoRV restriction enzyme site (underlined) was added to the reverse primer for ease of cloning. The *VSR1* promoter was cloned into the *pCAMBIA* vector via an SphI digest of *pGEM::VSR1* promoter and *pCAMBIA* followed by ligation and transformation into DH5α. The 35S

promoter was then replaced with the *GFP:GUS* fragment of *pBS::GFP:GUS* by a ClaI/PstI digest and ligation to make *pCAMBIA::VSR1promoter::GFP:GUS.*

VSR2promoter::GFP:GUS

The 2340 bp region upstream of the *AtVSR2* start codon was amplified with the following primers: Forward = ATA CTC ATC AAT GGA TAG CCA GC; Reverse = AAG CTT TCT TAT CAA GAA ACT TAT GTT TAG. A HindIII restriction enzyme site (underlined) was added to the reverse primer for cloning reasons. *pGEM::VSR2promoter* and *pCAMBIA::GFP:GUS* were digested with SacI (New England Biolabs) and ClaI (New England Biolabs) to replace the 35S promoter with the *VSR2* promoter to make *VSR2promoter::GFP:GUS.*

VSR3promoter::GFP:GUS

The 2160 bp region upstream of the *AtVSR3* start codon was amplified by PCR with the following primers: Forward = ATA AGC TTA ACG ACT ACT GCG TAT TGG AGA GC; Reverse = ATA AGC TTT GGA AGG TAA CAC AGA AGC TGC. HindIII restriction enzyme sites (underlined) were added for cloning purposes. *35S::GFP:GUS* and *pGEM::VSR3p* were digested with HindIII (New England Biolabs) and ligated to replace the 35S promoter with the *VSR3* promoter.

VSR4promoter::GFP:GUS

The 2100 bp region upstream of the *AtVSR4* start codon was amplified with the following primers: Forward = GCA TGC AAC CAC AAT TCA CGA AAC CCT AAT TTC; reverse = GGT ACC AAC AAA CAC CAA ATT CAA ACG GAT CAA C. SphI and KpnI restriction enzyme sites (underlined) were added to the ends of the primers for cloning purposes. *pCAMBIA 1300MCS* and *pGEM::VSR4promoter* were digested with

SphI (New England Biolabs). The *VSR4* promoter fragment was then ligated into *pCAMBIA*. The resulting construct and *pBS::GFP:GUS* were then both digested with SacI/KpnI (New England Biolabs) in order to replace the 35S promoter with the *GFP:GUS* fragment to make *VSR4promoter::GFP:GUS*.

VSR5promoter::GFP:GUS

The 2400 bp region upstream of the *AtVSR5* start codon was amplified with the following primers: forward = GCA TGC AGA CTT CAC ATA GAG ACG ATG GGA TG; reverse = GAT ATC TGA ACC TTA ATG TAT ACG GAA GAG ACG. SphI and EcoRV restriction enzyme sites (underlined) were added in the primers for cloning purposes. To make *VSR5promoter::GFP:GUS, pGEM::VSR5promoter* was digested with SphI and SalI; *pBS:::GFP:GUS* was digested with SalI/SacI (New England Biolabs); and *pCAMBIA 1300MCS* was digested with SphI/SacI (New England Biolabs) . The relevant fragments from each digest were joined in a 3-body ligation and transformed into DH5α.

VSR7promoter::GFP:GUS

The 2340 bp region upstream of the *AtVSR7* gene was amplified with the following primers: forward = GCA TGC AAG AAC ACT GTC AAT ACA CAA CAT G; reverse = AAG AAG AGT TTG ATC GAT GAT AAC C. An SphI restriction enzyme site (underlined) was added to the forward primer for cloning purposes. To make the *VSR7promoter::GFP:GUS, pGEM::VSR7promoter* was digested with PstI/ApaI (New England Biolabs); *pBS::GFP:GUS* was digested with ApaI/SacI (New England Biolabs); and *pCAMBIA 1300MCS* was digested with PstI/SacI. The relevant fragments from each digest were ligated in a 3-body ligation and transformed into DH5α.

Histochemical Staining of GUS

Plant tissue was stained following the protocol of Jefferson (Jefferson et al., 1987) with modifications. The stained tissues were cleared in 70% Ethanol and visualized by either the MZIII Dissection Microscope (Leica) or the Insight Confocal Microscope (Meridian). Micrographs were taken with a retail grade digital camera on the Leica or the Hamamatsu CCD camera on the Insight.

Expression Analysis of AtVSR3 Under Stress Conditions

Wildtype Columbia *Arabidopsis* plants were grown on agar containing Murashige Minimal Organics Medium (Invitrogen/Gibco) under constant light at 22°C for four weeks. The plants were then subjected to various stresses as indicated below following the published protocols (Yamaguchi-Shinozaki and Shinozaki, 1994). For cold treatment, the plants were transferred to fresh agar plates and placed in 23°C or in 4°C for the indicated time points.

For drought stress, the plants were transferred to either a fresh agar plate as a control or transferred to a piece of Whatmann filter paper in a Petri dish. Both plates were incubated at 23°C under low light conditions for the indicated time points.

For salt stress, the plants were transferred to liquid media containing Murashige Minimal Organics Medium with 250mM NaCl or to liquid media with an equal volume of sterile water as a control. For ABA treatment, the plants were transferred to liquid media with 250uM ABA or an equal volume of ethanol as a control. Flasks for both salt and ABA treatments were incubated on a rotary shaker for the indicated time points.

RNA was extracted from the indicated time points for all of the samples using the RNeasy Extraction Kit (Qiagen). 500ng-1ug of RNA was used as a template in One-Step RT-PCR (Qiagen) with gene-specific primers for *AtVSR3* (described above) or *ubiquitin* (forward = GAT CTT TGC CGG AAA ACA ATT GGA GGA TGG T; reverse = CGA CTT GTC ATT AGA AAG AAA GAG ATA ACA AGG). The PCR products were electrophoresed in a 1% agarose gel containing ethidium bromide and photographed with a Biorad Gel Documentation system (Biorad). Densitometry analysis of the PCR products was accomplished with the MCID Elite 6.0 software.

Results

Phylogenetic Analysis of the Plant VSR Gene Family Reveals Three Groups of VSRs

The *Arabidopsis* genome encodes seven putative VSRs that share high sequence identity (Hadlington and Denecke, 2000; Shimada et al., 2003). A gene family is a group of genes that share a common splicing pattern and likely arose from gene duplication (Sanderfoot et al., 2000). To determine whether the *Arabidopsis* putative VSR genes are members of the same gene family, I compared the splicing patterns of the *AtVSR* genes (Figure 2.1). AtVSRs 1,3,4,5, and 6 have identical patterns; *AtVSR2* has one extra intron and *AtVSR7* lacks what is the fifth intron in the other genes. Therefore, these are remarkably similar, indicating that the At*VSRs* arose from a single progenitor gene and thus are members of the same gene family. VSRs have been experimentally identified in *Arabidopsis*, pea, pumpkin, and *Vigna mungo* (Kirsch et al., 1994; Ahmed et al., 1997; Shimada et al., 1997; Tsuru-Furuno et al., 2001). VSRs were also found through

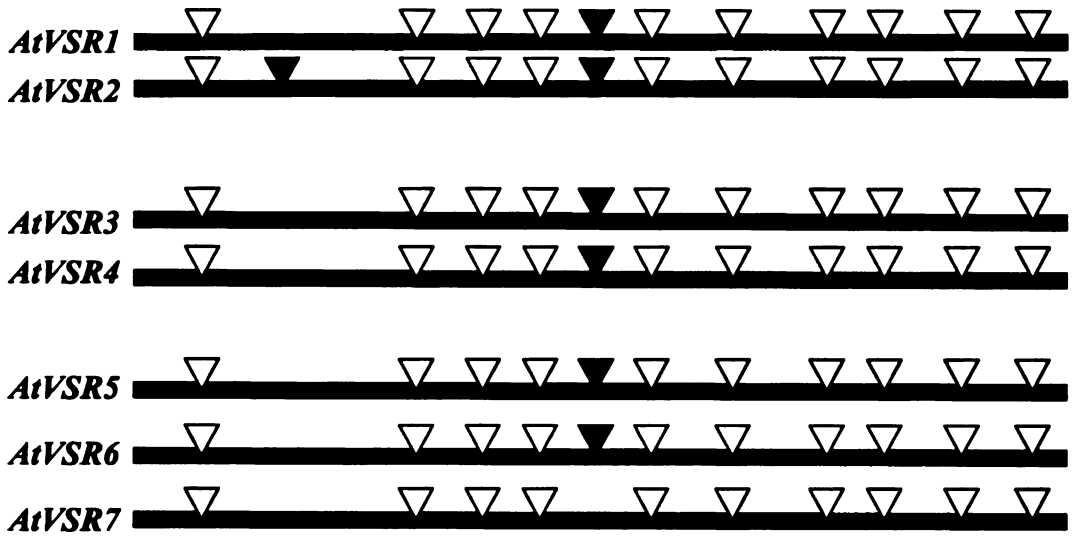


Figure 2.1. Gene structures of the *AtVSR* family. The schematic is a depiction of the cDNA and intron position for each gene. The black lines represent the cDNA for each gene. The white triangles represent the positions of introns that are found in the same location of each gene. The black triangles represent introns that are not found in all *AtVSR* genes.

bioinformatics in almond, maize, rice, sunflower, wheat, and *Physcomitrella patiens* (Table 2.1).

Table 2.1. Putative Plant VSRs

Organism	Accession or AGI Number/Name	Full Length Sequence Known?
Arabidopsis thaliana	At3g52850/AtVSR1	Yes
	At2g30290/AtVSR2	Yes
	At2g14740/AtVSR3	Yes
	At2g14720/AtVSR4	Yes
	At2g34940/AtVSR5	Yes
	At1g30900/AtVSR6	Yes
	At4g20110/AtVSR7	Yes
Pisum sativum (Garden Pea)	P93484/BP-80	Yes
Cucurbita (Pumpkin)	O48662/PV72	Yes
	PV82	No
Oryza sativa (Rice)	8352.m04840	Yes
	8362.m01486	Yes
	8360.m02012	Yes
	8354.m04241	No
	8354.m04240	No
	8355.m04617	No
Physcomitrella patens	Q9AWB4	No
Helianthus annuus (Sunflower)	Q94IN3	No
	Q94IN4	No
	Q9ARG7	No
Prunus dulcis (Almond)	Q9SDR8	No
Zea mays (Maize)	P93645	No
Vigna mun (Black gram)	Q93X09	Yes
Triticum aestivum (Wheat)	Q9LLR3	Yes

To understand how the VSRs are related, I determined the phylogenetic relationship between these proteins based on their amino acid sequences. The phylogenetic relationship of these proteins for which full length sequence was known was calculated with PHYLIP, a software package that determines the relatedness of a group of proteins and uses a midpoint method to determine the root of the tree (Felsenstein, 1988). The results indicated that the VSRs separated into three groups (Figure 2.2)

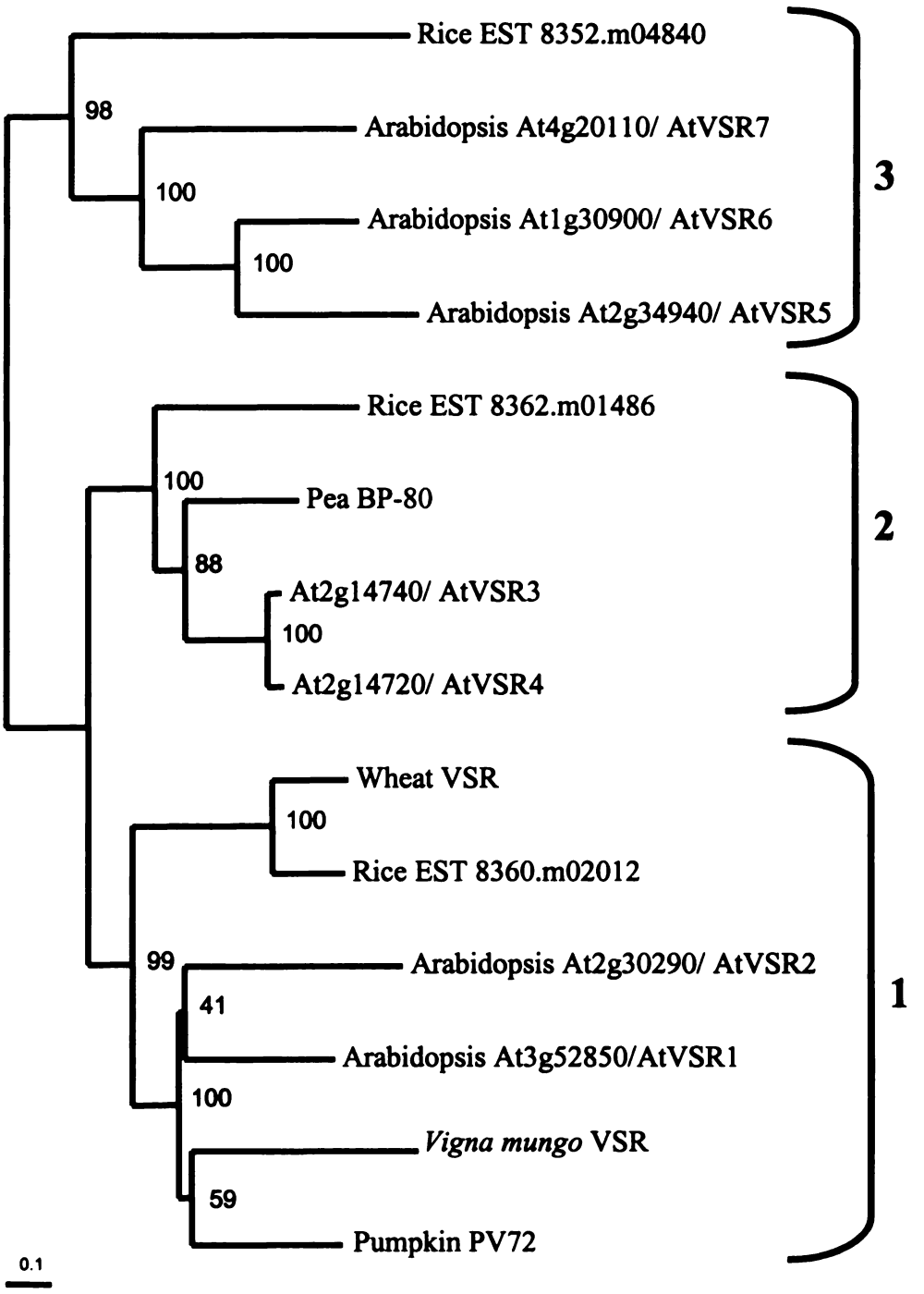


Figure 2.2. Phylogenetic analysis of the plant VSRs. Phylogenetic analysis indicated that the family of VSRs clusters into three groups. The full length amino acid sequences were analyzed to determine their relationships to one another. The tree was calculated with the PHYLIP package (Felsenstein 1989) using the PROTDIST program for calculating the distance matrix (categories model as distance matrix), the NEIGHBOR-JOINING method for tree construction and the midpoint method in RETREE for defining the root of the trees. The internal bootstrap values were obtained from 100 alignment re-sampling replicates.

(Paris and Neuhaus, 2002). The first group contained AtVSR1 and PV72, among other proteins. The second group was composed of pea BP-80 as well as other proteins. The third group was composed of three uncharacterized putative AtVSRs and two putative rice VSRs.

All putative plant VSRs have a PA domain that is upstream of the cysteine-rich EGF repeats (Mahon and Bateman, 2000; Shimada et al., 2003). The PA domain is thought to be the site of protein-protein interaction for proteins (Mahon and Bateman, 2000). Moreover, the NTPP-interaction domains of BP-80 and PV72 are N-terminal to the cysteine-rich repeats (Cao et al., 2000; Watanabe et al., 2002). Therefore, it is possible that the PA domain is the site of cargo protein interaction (Mahon and Bateman, 2000). I examined the phylogenetic relationship of the VSRs using only the amino acid sequence from the PA domain of each VSR for which the full length protein sequence was known (Figure 2.3). The phylogeny of the PA domain of the VSRs was identical to the phylogeny of the entire VSRs. This information suggested that the relatedness of the plant VSRs could be indicative of their function. To address the functions of the AtVSRs, I looked at the expression patterns for each *Arabidopsis VSR* gene.

Expression Patterns of AtVSR1 and AtVSR, Members of the Group 1 VSRs

AtVSR1 and AtVSR2 proteins are members of the first group of plant VSRs (Figure 2.2). To determine the expression patterns of *AtVSR1* and *AtVSR2,* RT-PCR was carried out with gene-specific primers using total RNA from various *Arabidopsis* tissues as a template. Each PCR was limited to less than 28 cycles to stay within a linear range

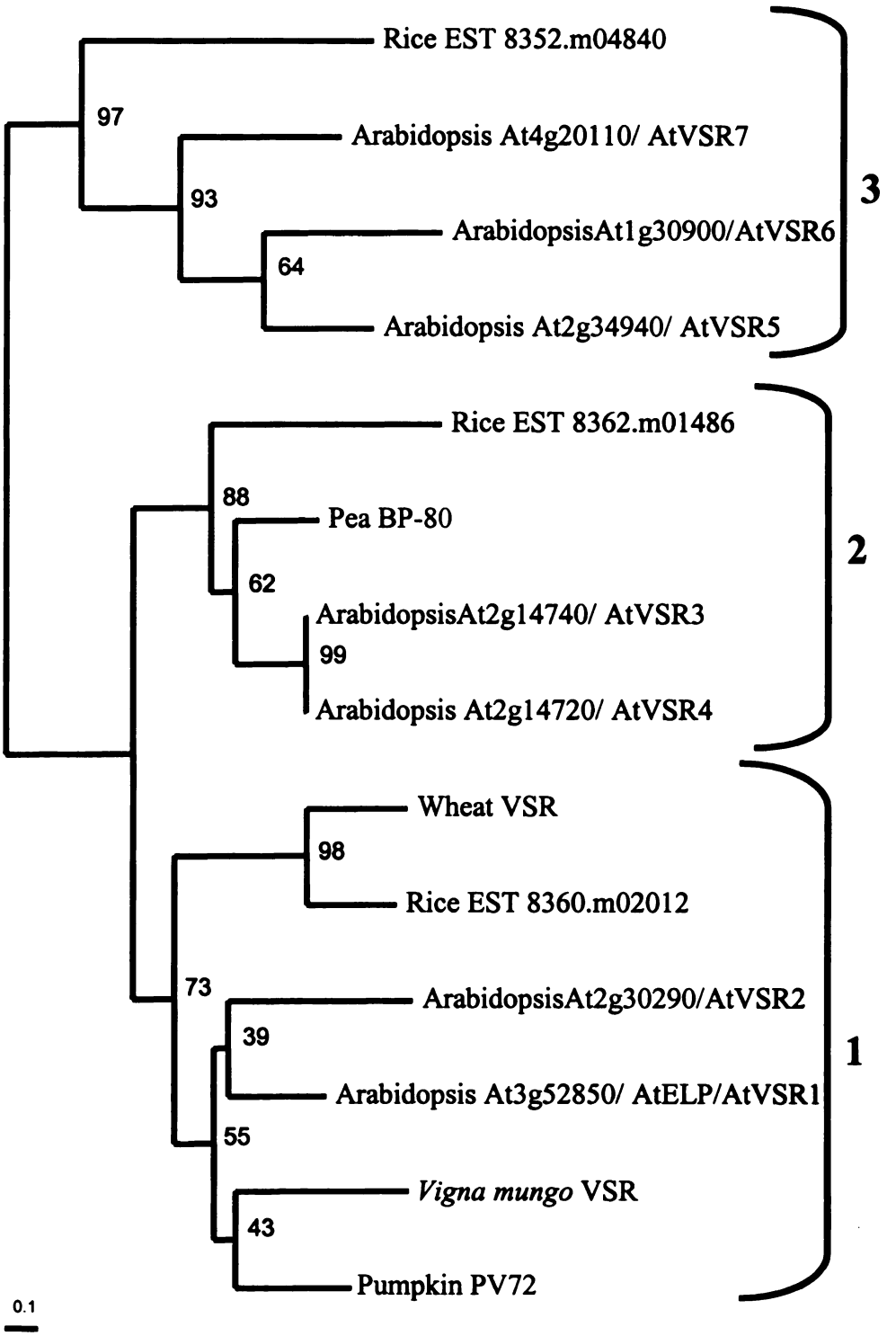


Figure 2.3. Phylogenetic analysis of the PA domain of the plant VSRs. Phylogenetic analysis of the PA domain of plant VSRs indicated that the putative ligand binding domain of the family of VSRs clusters into three separate goups. The amino acid sequence of the PA domains of the plant VSRs were compared to determine their relationships to one another. The tree was calculated as described in Figure 2.2.

of amplification and thus obtain a more accurate picture of the relative levels of gene expression in the various tissues (Figure 2.4). *AtVSR1* expression was detected in all tissues examined (Figure 2.4). However, the expression of *AtVSR2* was restricted mostly to the roots plus a barely detectable amount present in young leaves (Figure 2.4). The difference in expression patterns between *AtVSR1* and *AtVSR2* suggested that the functions of these two genes were not redundant.

The widespread expression pattern of *AtVSR1* led to the question of whether *AtVSR1* was simply a ubiquitously expressed housekeeping gene or whether it was expressed in specific cell types throughout the plant. I was also interested in confirming the root localization of *AtVSR2*. To answer these questions, I made DNA constructs that contained the putative promoter region for each gene fused to the coding region of *β-glucuronidase* (*GUS*). The transcriptional fusions were transferred to a binary vector for stable transformation in *Arabidopsis*. The constructs were transformed into *Arabidopsis*, and putative transformants were selected for resistance to hygromycin and propagated. The T2 and T3 generations were screened for GUS activity.

Five transgenic lines were selected for analysis by GUS assay for the *AtVSR1* promoter. GUS activity showed that *AtVSR1* was expressed in the mature embryo of imbibed seeds, as well as the meristematic region and vasculature of seedlings (Figure 2.5A-D). In mature plants, GUS protein was detected throughout the vascular system of roots, leaves, and inflorescences (Figure 2.5E-G). GUS was also detected in the flower carpel as well as in the maturing embryo from an immature silique (Figure 2.5H,I). These results were consistent with the results obtained by RT-PCR (Figure 2.4), but suggested that AtVSR1 may have a more specific function than a house-keeping protein.

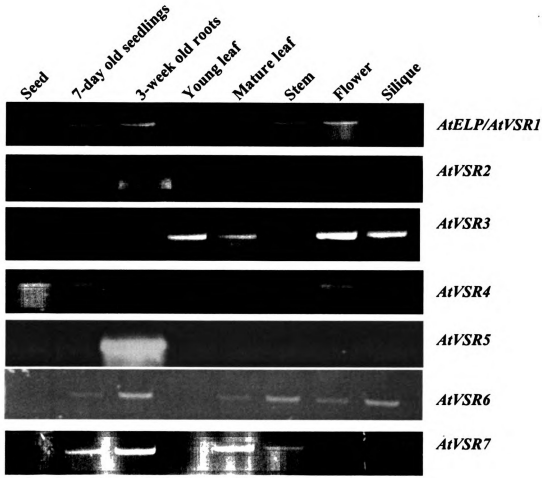
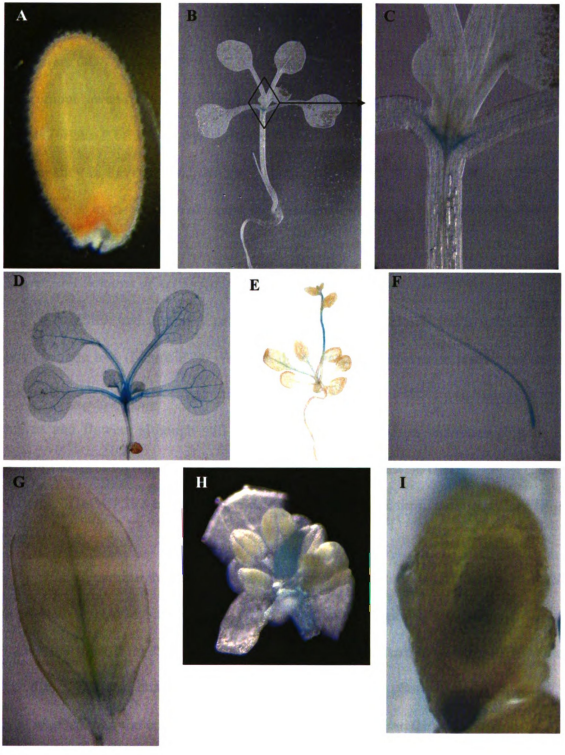


Figure 2.4. RT-PCR expression analysis of *AtVSR* genes. RNA was extracted from various Arabidopsis tissues as indicated in the figure. 500 ng to 1 ug of RNA was used as a template in RT-PCR with gene-specific primers and 20-25 cycles of PCR after reverse transcription cDNA synthesis.

Figure 2.5. Expression analysis of *AtVSR1* by Promoter::GUS fusions in *Arabidopsis*. *AtVSR1 promoter::GUS* transgenic plants were histochemically stained with the X-Glucuronide substrate to visualize the tissue and cell-type specific expression pattern of *AtELP/AtVSR1*. GUS activity is seen the mature embryo (A). GUS activity is also detected in the meristematic region of 7-day old seedlings (B and C). 2-week old plants and 4-week old plants express GUS throughout the vascular tissue (D and E, respectively). Closer examination of the leaves and the roots confirmed that the GUS activity is localized to the vascular tissue (F, G). In flowers, GUS is detected in the carpel (H). GUS is also detected in maturing embryos from green siliques (I).


A
B
C
D
E
F
G
H
I

Eleven *AtVSR2 promoter::GUS* lines from three independent transformations were analyzed to gain more insight into the expression pattern of *AtVSR2*. In agreement with the RT-PCR results, GUS activity was detected in the roots (Figure 2.6E). However, GUS was also detected in the cotyledons of mature embryos (Figure 2.6A), throughout seven-day old seedlings (Figure 2.6B), in the vasculature of four-week old plants (Figure 2.6C,D), as well as in the flowers of transgenic plants (Figure 2.6E). The discrepancy in the expression patterns obtained by RT-PCR and *promoter::GUS* fusion could be due to the absence of a repression element(s) in the *promoter::GUS* fusion.

Expression Patterns of AtVSR3 and AtVSR4, Members of the Group 2 VSRs

AtVSR3 and AtVSR4 proteins are part of the second group of the AtVSR phylogenetic tree (Figure 2.2). We first determined the expression patterns of *AtVSR3* and *AtVSR4* by RT-PCR (Figure 2.4). The expression of *AtVSR3* was highest in young leaves and flowers although still detected in all other tissues examined (Figure 2.4). *AtVSR3* expression pattern was detected in seeds with at least 28 cycles of PCR (data not shown). The *AtVSR4* transcript was detected at low levels throughout the plant (Figure 2.4).

To determine the cell type-specific expression patterns of *AtVSR3* and *AtVSR4*, we made reporter fusions to the predicted promoter regions of each gene. Fifteen transgenic lines from three independent pools of plants carrying the *AtVSR3* promoter fused to *GUS* were analyzed. The GUS activity of these plants indicated that *AtVSR3* was expressed in the cotyledons of imbibed seeds, as well as the roots, hypocotyls, and cotyledons of seven-day old seedlings (Figure 2.7A-C). Surprisingly, expression from

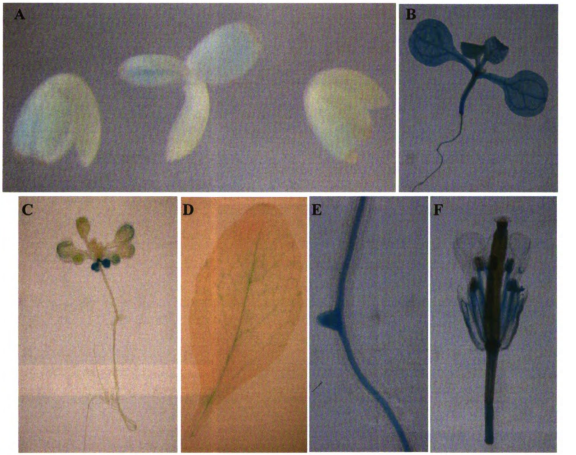


Figure 2.6. Expression patterns of *GUS* from the *AtVSR2* promoter. *AtVSR2 promoter::GUS* transgenic plants were histochemically stained with the X-Glucuronide substrate to visualize the tissue and cell-type specific expression pattern of *AtVSR3*. GUS activity was detected in the cotyledons of mature embryos (A) and throughout a seven-day old seedling (B). However, four-week old plant only showed GUS activity in the vasculature of the true leaves (C, D) and roots (E). GUS activity was also detected in the sepals, stamens, and carpel of the flower (F).

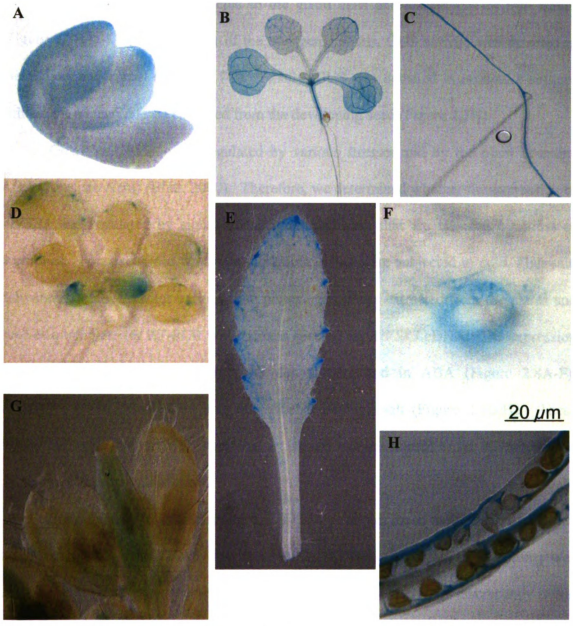


Figure 2.7. Expression analysis of *AtVSR3* by Promoter::GUS fusions in *Arabidopsis*. *AtVSR3* promoter::GUS transgenic plants were histochemically stained with the X-Glucuronide substrate to visualize the tissue and cell-type specific expression pattern of *AtVSR3*. GUS activity was detected in the cotyledons of mature embryos (A). 7-day old seedlings (B) express GUS throughout the cotyledons, hypocotyl, and primary root (C), although restricted to the margins of the true leaf. 4-week old plants (D) show similar results of GUS expression throughout the cotyledons that is primarily restricted to the hydathodes in the true leaves (D, E). Upon closer examination of the rosette leaf (40X magnification) revealed GUS activity specifically in the guard cells (F). In the flowers, GUS is detected primarily in the carpel, but can be faintly seen in the anthers as well (G). GUS was not detected in the maturing embryos of green siliques (H), although it was present throughout the maternal tissues of the silique.

the *AtVSR3* promoter was restricted to the guard cells and hydathodes of true leaves (Figure 2.7B,D-F). In the flower of the transgenic plants, GUS activity was detected in the anthers and carpel (Figure 2.7G). Finally, GUS was detected in immature siliques although it appeared to be excluded from the developing seed (Figure 2.7H).

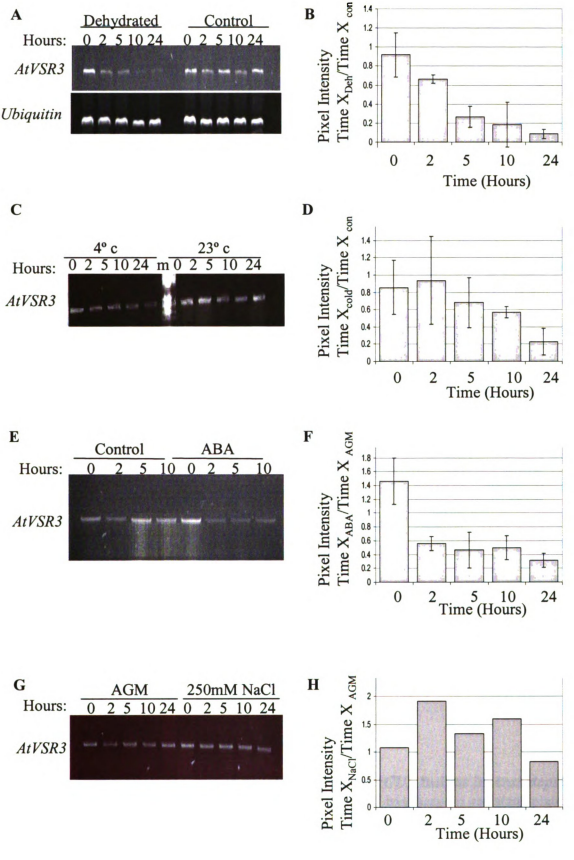
Guard cell function is regulated by various stresses and by the plant hormone ABA (Abscisic Acid) (Blatt, 2000). Therefore, we determined whether the expression of *AtVSR3* was regulated by signal transduction pathways that are initiated by stress or hormones. Four-week old wild type Columbia plants were subjected to cold, high salt, dehydration, and ABA for various time points. RNA was extracted from the plants and used as a template for RT-PCR with primers specific for *AtVSR3* cDNA. The expression of *AtVSR3* was repressed under cold, dehydration, and in ABA (Figure 2.8A-F). However, *AtVSR3* expression was not affected by high salt (Figure 2.8G,H). These results indicated that *AtVSR3* is affected by certain stresses related to the ABA response pathways, but not by all of them.

Eight transgenic lines carrying the *AtVSR4* promoter fused to *GUS* were analyzed to determine the expression pattern from the *AtVSR4* promoter. No GUS activity was detected in any of the transgenic plants (Figure 2.9). Most likely, the discrepancy is that the promoter::GUS fusion construct does not encode the complete *AtVSR4* promoter or the construct is missing intron-encoded regulatory elements.

Expression Patterns of AtVSR5, AtVSR6, and AtVSR7, Members of the Group 3 VSRs

AtVSR5, AtVSR6, and *AtVSR7* belong to the third group of VSRs. The third group was composed of putative VSRs that have not been characterized at the protein level. We

Figure 2.8 *AtVSR3* Expression decreases in the presence of dehydration, cold, or 250 uM ABA. *Arabidopsis* plants were grown on agar for four weeks. The plants were then transferred to either fresh agar plates or petri dishes with filter paper for the indicated time points (A, B); transferred to fresh plates and kept at 4°c or 23°c for the indicated time points (C, D); or were transferred to liquid media in the presence or absence of 250uM ABA for the indicated time points (E, F). RNA was extracted and 1 ug total RNA template was used in 25 cycles of RT-PCR with primers specific to *AtVSR3* or to *ubiquitin*. The intensity of each band was measured for the treatment and the control. Within each experiment, the subtracted pixel intensity of the treated sample was divided by the subtracted pixel intensity of the untreated sample for the same time point. The average of three independent experiments and their relative standard deviations were graphed in the chart.


A
Dehydrated
Control
Hours: 0 2 5 10 24 0 2 5 10 24
AtVSR3
Ubiquitin
B
Pixel Intensity
Time X_Deh/Time X_con
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C
4° c
23° c
Hours: 0 2 5 10 24 m 0 2 5 10 24
AtVSR3
D
Pixel Intensity
Time X_cold/Time X_con
Time (Hours)
E
Control
ABA
Hours: 0 2 5 10 0 2 5 10
AtVSR3
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Pixel Intensity
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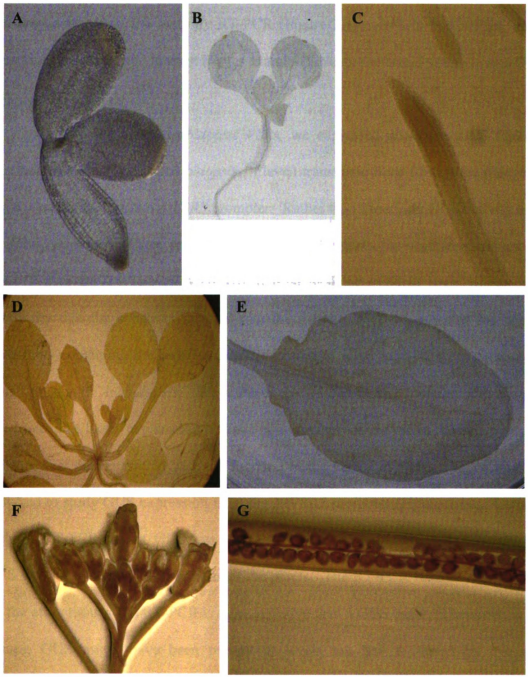

Figure 2.9. Expression analysis of *VSR4* by *Promoter::GUS* fusions in *Arabidopsis*. GUS activity was not detected in any cells examined of hygromycin resistant plants, including mature embryos (A), 7-day old seedlings (B), roots (C), 4-week old plants (D), rosette leaves (E), flowers (F), and immature siliques (G).

first examined the expression patterns of these genes by RT-PCR. *AtVSR5* expression was only detected in the roots by RT-PCR (Figure 2.4). *AtVSR6* and *AtVSR7* mRNA transcripts were detected in most tissues examined except seeds and young leaves (Figure 2.4).

As with the other *Arabidopsis VSRs,* we expressed *promoter::GUS* fusions of each putative promoter in *Arabidopsis*. Eleven transgenic lines from three independent pools were studied for the *AtVSR5* promoter. Rather than observing GUS activity only in the roots, we observed very strong GUS staining throughout the vegetative tissues of the plant (Figure 2.10). The effect was seen in several independent lines, ruling out the possibility that the construct inserted into the genome near an enhancer for one line. Constructs containing the putative *AtVSR6* promoter fused to *GUS* were prepared and transformed into *Arabidopsis*. We were unable to obtain any transformants from this construct. The likely explanation is that a point mutation occurred in the binary vector of the construct that interfered with the function of the vector in plants. Seven lines were analyzed to study GUS expression from the AtVSR7 promoter (Figure 2.11). Consistent with the RT-PCR results, GUS was detected at the distal ends of leaves and in the roots. GUS was not detected in other parts of the plants. The RT-PCR results were very faint for the other tissues and RT-PCR is more sensitive than a GUS assay. Therefore, in other tissues, GUS might have been present at levels too low to detect by eye or by microscopy.

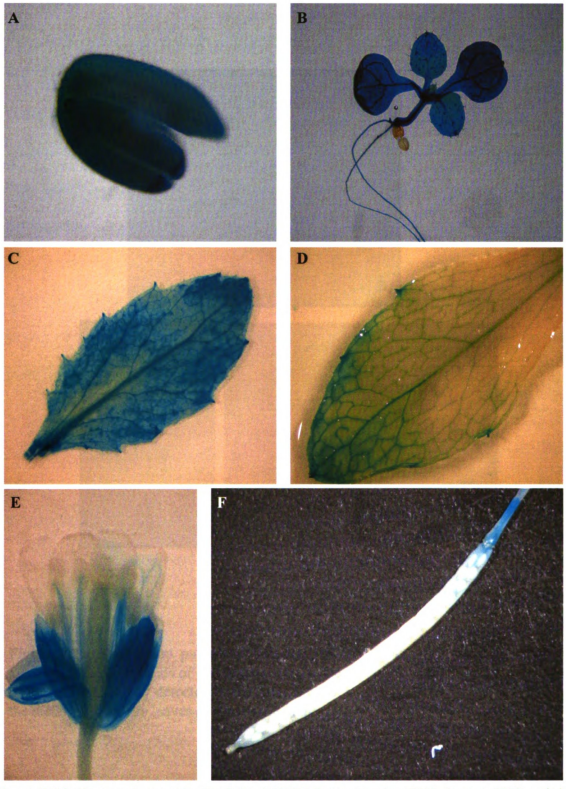

Figure 2.10. Expression analysis of the*AtVSR5* Promoter by GUS Assay. GUS activity was strongly detected in the mature embryo (A), the seven-day old seedling (B), and the rosette leaf (C). GUS was detected in the distal end of cauline leaves (D), the sepals, petals, filament and carpel of the flower (E), but only at the base of the silique (F).

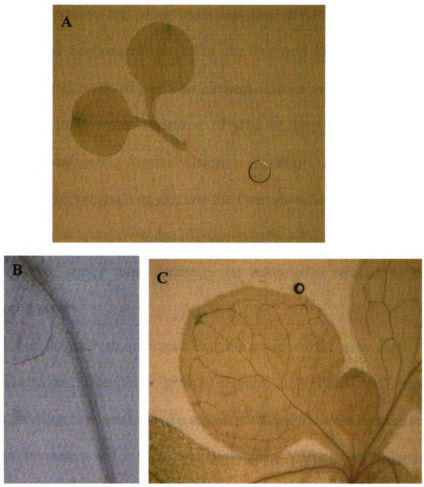

Figure 2.11. Expression patterns from the *AtVSR7* Promoter. GUS activity was detected at the distal ends of cotyledons and the root-shoot transition zone of seedlings (A). GUS was faintly detected in the roots and root hairs (B). GUS was detected in the distal ends of true leaves of the rosette plant (C).

Discussion

Three approaches were taken to determine the expression patterns of members of the *AtVSR* gene family, including bioinformatics, RT-PCR, and promoter::reporter fusions. Expressed Sequence Tags (EST), the Stanford Microarray Database (SMD), Affymetrix Chip, and predicted promoter element data are available to the public through The *Arabidopsis* Information Resource (TAIR) at http://www.arabidopsis.org/, the Nottingham *Arabidopsis* Stock Centre (NASC) Affymetrix Database at http://www.ssbdjc2.nottingham.ac.uk/; and the Plant cis-acting regulatory DNA elements (PLACE) database at http://www.dna.affrc.go.jp/htdocs/PLACE/signalscan.html/ (Higo et al., 1999). We mined these databases for information relating to the expression patterns of each gene.

Phylogenetic analysis indicated that the plant *AtVSR* gene family can be sub-divided characterized into three groups (Figure 2.2) (Paris and Neuhaus, 2002). Furthermore, phylogenetic analysis of a region predicted to be the domain responsible for cargo protein interaction that is present in all the plant VSRs gave similar results (Figure 2.3). From this information, we hypothesized that there were three functional groups within the VSR family. A summary of the observations made in this chapter is presented in Table 2.2.

Group 1 VSRs

The first group is composed of PV72, AtVSR1, a VSR identified in the ER bodies of *Vigna mungo*, and proteins from *Arabidopsis*, rice, and wheat. Previous reports

Table 2.2 Summary of the Spatial and Temporal Expression Data Obtained for the *AtVSR* Genes

Gene	Seeds				Seedlings			Roots				Leaf				Flowers			Sugar Starvation		Phosphate Starvation
	RTPCR	GUS	EST/MA/AC[c]	Promoter elements[a]	RTPCR	GUS	EST/MA/AC[c]	RTPCR	GUS	EST/MA/AC[c]	Promoter Elements[a]	RTPCR	GUS	EST/MA/AC[bc]	Promoter Elements[a]	RTPCR	GUS	EST/MA/AC[c]	EST/MA/AC[c]	Promoter Elements[a]	EST/MA/AC[c]
AtVSR1	X	X	8	23	X	X	1	X	X		5	X	X	X	1	X	X				
AtVSR2		X				X		X	X		26	X	X				X	X[d]			
AtVSR3	X	X	4	1	X	X		X	X	2		X	X[b]	X[b]	25[b]	X	X				
AtVSR4	X		1	5	X			X		1		X				X					
AtVSR5								X	X	2				X	X	X	X				
AtVSR6	X				X			X				X		X					X	4	
AtVSR7	X				X			X	X			X		X		X		X			X

[a]Promoter elements are described in greater detail in Appendix A

[b]Expression was restricted to the guard cells

[c]EST=Expressed Sequence Tag; MA=microarray; AC=Affymetrix Chip

[d] *AtVSR2* was specifically detected in the pollen (Becker et al., 2003).

suggest that PV72, AtVSR1, and the *Vigna mungo* VSR function, at least in part, as VSRs for soluble proteins that are destined for the PSV (Shimada et al., 1997; Tsuru-Furuno et al., 2001; Shimada et al., 2003). Therefore, it is possible that the first group of VSRs primarily function in the delivery of storage proteins to the PSV. Expression analysis of *AtVSR1* indicated that it was expressed in developing and mature seeds as well as throughout the vasculature of the mature plant. ESTs for *AtELP/AtVSR1* were found in developing seeds, green siliques, 3-day old seedling hypocotyls, and dehydrated rosette plants (Table A.1). The presence of seed and seedling ESTs for *AtVSR1* was consistent with our RT-PCR and GUS results. Five ESTs for *AtVSR1* were also identified in dehydrated plants (Table A.1). Furthermore, microarray data suggested that *AtELP/AtVSR1* expression was also regulated by ABA (SMD experiments 11895, 11757).

An analysis of the *AtVSR1* promoter by the PLACE Signal Scan Database (Higo et al., 1999) revealed promoter elements for expression in endosperm, embryo, and mature seeds (Table A.2). In particular, the *AtVSR1* promoter contains a combination of the GCN4 motif, the AACA motif, and the ACGT motif in close proximity to each other (Table A.2). The combination of these three motifs in a short region is necessary to activate expression in endosperm (Wu et al., 2000). A second combination of *cis*-elements found in the *AtVSR1* promoter that enhances expression in seeds was the combination of the SEF3 (soybean embryo factor) element, the SEF4 element, and the RY element (Table A.2) (Lessard et al., 1991; Fujiwara and Beachy, 1994). A number of other elements that activate or enhance expression in seeds were also found in the *AtVSR1* promoter such as the -300 element (one copy at -10), the TATCCA motif (four

copies), the DPBF (Dc3 promoter-binding factor) consensus motif (two copies), and the E-Box (10 copies) (Table A.2). The -300 element is a promoter element that is consistently found within the first 300 bases upstream of the start codon of seed storage genes (Thomas and Flavell, 1990). The DPBF consensus motif and the E-Box enhance expression in seeds (Kim et al., 1997). The possibility that these are functional elements in the *AtVSR1* promoter is supported by the RT-PCR and GUS data that indicated that *AtVSR1* is expressed in developing and mature embryos. Taken together, these observations further suggested that AtVSR1 plays a role in processes related to seed development and certain stresses. PSVs accumulate during both of these processes (Paris et al., 1996; Jauh et al., 1999; Park et al., 2004). Therefore, the expression data obtained for *AtVSR1* were consistent with the model that AtVSR1 functions in the transport of proteins to the PSV and that AtVSR1 is important for seed development.

RT-PCR and promoter::reporter studies demonstrated that the *AtVSR2* gene is expressed in the roots. However, the RT-PCR data indicated that *AtVSR2* was only expressed in the roots, whereas analysis of transgenic plants containing the putative *AtVSR2* promoter fused to *GUS* suggested that *AtVSR2* is expressed in other tissues as well. The promoter::reporter transgenic plants were generated by transforming three separate pots of wildtype *Arabidopsis* plants with multiple plants per pot. While it was possible that the construct entered into the genome next to an expression enhancer for one line, it was unlikely that a similar event would occur for at least three independent lines. Restriction digest and DNA sequencing analysis confirmed that the construct did not have any part of the 35S promoter and did have the putative *AtVSR2* promoter (Data not shown). Promoter analysis through the PLACE Database (Higo et al., 1999) revealed 26

copies of a promoter element that activates expression in the roots (Table A.3) (Elmayan and Tepfer, 1995). Therefore, it is possible that the high expression in roots is due to the 26 copies of this root expression promoter element.

The *AtVSR2::GUS* plants also showed weak GUS activity in the cotyledons of seeds and in seedlings. In agreement with this data, the *AtVSR2* promoter encodes many seed expression elements (Table A.3). One of these elements is a version of the RY element that is less able to suppress expression in leaves than another version of the RY element (Fujiwara and Beachy, 1994). Perhaps this is why there is GUS activity in the cotyledons and the vasculature of the leaves of the transgenic plants that was not accounted for by RT-PCR. Finally, Affymetrix studies of the pollen transcriptome indicate that *AtVSR2* expression in the pollen is 2.8 times higher than in leaves, roots, seedlings, and siliques (Becker et al., 2003). I also saw *AtVSR2* expression in the pollen in the promoter::GUS fusion studies. The vacuole is a dynamic and important compartment in pollen (Hicks et al., 2004). Therefore, it is possible that AtVSR2 participates in pollen growth. Overall, these results were not strong indicators as to whether AtVSR2 is also a receptor for storage proteins. However, α-TIP is detected in roots and leaves, suggesting that PSVs are also present in those tissues (Paris et al., 1996; Park et al., 2004). While it is possible that AtVSR2 is the VSR for storage proteins, this hypothesis must be addressed by a biochemical or reverse genetics approach.

Group 2 VSRs

The second group of VSRs was composed of the previously characterized BP-80 from pea, two homologues from *Arabidopsis* (*AtVSR3* and *AtVSR4*), and a putative VSR

from rice. BP-80 was identified as a VSR for the CCV pathway and is strictly localized to CCVs relative to dense vesicles (Kirsch et al., 1994). Thus, members of this group may be responsible for soluble proteins that are delivered to the lytic vacuole by the CCV pathway.

The expression pattern of *AtVSR3* is perhaps the most intriguing of the *AtVSRs*. *AtVSR3* is expressed in mature embryos and ubiquitously expressed in seven-day old seedlings. However, beginning with the first true leaves, expression is restricted to the guard cells and the hydathodes until flowering, when GUS activity is also detected in the anthers and the carpels. Further RT-PCR analysis indicated that *AtVSR3* is repressed by ABA, dehydration, and cold treatment. Affymetrix data also indicated that *AtVSR3* is specifically expressed in guard cells and is repressed by ABA (Leonhardt et al., 2004). An analysis of the putative *AtVSR3* promoter revealed that the *AtVSR3* promoter has 25 copies of the DOF (DNA-binding with one finger) motif that has been observed in the promoters of other guard cell-specific genes and promoter elements for ABA-mediated repression (Table A.4) (Higo et al., 1999; Plesch et al., 2001; Mena et al., 2002). The endomembrane system has many components that have been implicated in regulation of stomatal movement (Blatt, 2002; Geelen et al., 2002; Zhu et al., 2002). It is exciting to speculate that AtVSR3 is the VSR for a vacuolar protein that functions in keeping the stomata open through some function in the vacuole. When the plant is exposed to cold or drought stress, the stomata close, and the ABA signal transduction pathway represses the expression of *AtVSR3* and presumably represses the expression of the specific cargo protein as well.

AtVSR4 expression was detected in all tissues by RT-PCR. However, GUS assays of transgenic promoter::reporter plants did not reveal any GUS activity at all. The region of DNA between *AtVSR4* and its immediately adjacent gene, *At2g14730*, is only 500 bases and only this region was used to prepare the *promoter::GUS* construct. *At2g14730* is a hypothetical protein of unknown function for which no ESTs have been identified. It is possible that the promoter of the *AtVSR4* gene actually extends farther into the *At2g14730* gene. Therefore, the likely possibility is that the promoter for *AtVSR4* is longer than predicted and therefore, only part of the promoter is present in the reporter fusion construct. ESTs for *AtVSR4* have been identified in developing seeds (Accession BE25715), roots (AV545281), and dehydrated rosette plants (AV795948), confirming that *AtVSR4* is expressed. Some promoter elements for seed expression are present in the 1500 bases upstream of the *AtVSR4* start codon (Table A.5). Thus, the expression pattern of *AtVSR4* is similar to *AtVSR1*. Based on the phylogenetic tree of the *AtVSR* gene family, it is possible that AtVSR4 is performing a distinct function from AtVSR1 in the same tissues.

Group 3 VSRs

The third group of VSRs was composed of proteins that have not been studied previously. RT-PCR demonstrated that *AtVSR5* expression only occurs in the roots. Contradictory to this data, the GUS assays showed ubiquitous expression of GUS from the predicted promoter of *AtVSR5*. Transcriptional and post-transcriptional regulatory *cis*-elements can exist in the introns as well as in the 3'UTR (untranslated region) of genes. Therefore, it is likely that suppressor elements of *AtVSR5* exist outside of the

promoter region. The effects of these elements would be seen in the RT-PCR results, but would be masked in the GUS assays since only the upstream sequences were used in the reporter fusion. An analysis of the introns and 3'UTR of *AtVSR5* manually and by the PLACE database did not reveal any characterized repression elements. The 3'UTR has two copies of the AU-Rich Element (ARE) that confers mRNA instability to a gene. However, the ARE is usually present much more abundantly in previously characterized mRNAs (Gutierrez et al., 1999). Therefore, the most likely explanation is that there was negative *cis*-regulatory information present in the introns or the 3'UTR of the *AtVSR5* gene. The absence of the negative regulators would allow expression from the *AtVSR5* promoter to occur uninhibited. To this end, it would be interesting to examine the expression pattern of different portions of the entire *AtVSR5* gene by reporter fusions to identify such elements. Bioinformatic data was very limited for *AtVSR5*. However, in agreement with the RT-PCR results, the only ESTs that have been identified were both from root tissue (Accessions AU235566 and AU226294).

RT-PCR of *AtVSR6* indicated that it is expressed in most vegetative tissues with the exception of young leaves. I was not able to recover any transgenic plants for promoter analysis. One microarray indicated that *AtVSR6* may be more highly expressed in response to starvation (D. Bassham, personal communication). Consistent with this, the promoter of *AtVSR6* contains five promoter elements that are thought to be starvation and/or Gibberellic Acid (GA) response elements in the α-amylase promoter (Table A.6). Perhaps one of the roles of AtVSR6 is to help the plant to adapt to starvation conditions. Other endomembrane trafficking proteins are involved in plant adaptation to starvation,

such at AtVTI12 (Surpin et al., 2003). Therefore, it would be interesting to examine the possible interactions between AtVTI12 and AtVSR6.

RT-PCR indicated that *AtVSR7* was expressed in seedlings, roots, and mature leaves. This was confirmed by promoter::reporter analysis. Furthermore, the GUS assays indicated that AtVSR7 expression in leaves was restricted to the distal end. Microarray data indicated that AtVSR7 expression increased in response to phosphate starvation (Hammond et al., 2003). We did not find any promoter elements that corresponded with these expression patterns. AtVSR7 may have a very specific role in plant adaptation to phosphate starvation. Therefore, the third group of VSRs may help the plant adapt to changing environmental conditions.

With these and previously published results in mind, we can hypothesize that the VSRs cluster into three functional groups. The first group is responsible for protein transport to PSVs. The second group is responsible for CCV trafficking to the lytic vacuole. The third group helps the plant adapt to specific environmental conditions. From the hypothesis we can predict that the subcellular localization of the AtVSR protein should be indicative of its function. For example, if AtVSR1 is implicated in the transport of storage proteins to vacuoles by the dense vesicle pathway, then AtVSR1 protein should primarily be localized to dense and /or PAC vesicles. Likewise, the BP-80 group of AtVSRs should primarily localize to CCVs. However, we can not expect that a specific VSR will only be found in a particular type of vesicle because there is often crosstalk among the different pathways to the vacuole (Hoh et al., 1995). Another prediction would be that mutants of an individual VSR that did not produce functional protein of a specific AtVSR would have a phenotype indicative of its specific function.

For example, if a member of the third group of VSRs is involved in helping the plant to cope with a specific stress such as starvation, then a null mutant of that VSR would be less able to cope with starvation than a wildtype plant. The next steps are to use reverse genetic and biochemical approaches to test these predictions. Overall, the expression patterns of the individual *AtVSRs* were consistent with the predicted function of each group based on previous reports and our own phylogenetic analysis.

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Chapter 3

Functional Analysis of the AtVSR Family

Abstract

Soluble vacuolar proteins are delivered to the vacuole by vesicle trafficking through the endomembrane system. First, the proteins are cotranslationally inserted into the endoplasmic reticulum and transported to the Golgi. At the *trans*-Golgi Network (TGN), a vacuolar sorting signal (VSS) is recognized by the vacuolar sorting machinery and the protein is delivered to the vacuole. Three types of VSSs have been identified in plants. One type of VSS, the amino-terminal propeptide (NTPP), is an amino acid sequence that is cleaved from the mature protein after delivery to the vacuole. The NTPP has a conserved motif that is recognized by a vacuolar sorting receptor (VSR) in a sequence-specific manner. The VSR is a type I transmembrane protein with a large lumenal domain and a short cytoplasmic tail. Previous studies have indicated that VSRs interact with the NTPP signal at the TGN. Then, both proteins are packaged into clathrin-coated vesicles and are delivered to the PVC. The cargo and receptor dissociate in the acidic environment of the PVC and the receptor is recycled to the TGN whereas the cargo is delivered to the vacuole. However, other members of the VSR family have been identified in endomembrane vesicles outside of this pathway. Therefore, it is likely that the model of VSR function needs to be expanded to accomodate other possible vacuolar trafficking pathways. The *Arabidopsis* genome encodes seven *VSRs,* all of which are all expressed in diverse tissues at various points in the plant life cycle. I took a reverse genetics approach to determine the overlapping and individual functions of the *AtVSRs* by using antisense technologies and T-DNA insertion lines. Determining the functions of the AtVSR family will lead to a greater overall understanding of protein trafficking through the endomembrane system.

Introduction

Protein targeting to the vacuole occurs via vesicle trafficking through the endomembrane system. Three types of vacuolar sorting signals have been identified in plants: *i)* the N-terminal propeptide (NTPP), *ii)* the C-terminal propeptide (CTPP), and *iii)* the internal signal (Surpin and Raikhel, 2004). The NTPP is recognized by a vacuolar sorting receptor (VSR) that mediates its delivery to the vacuole via the prevacuolar compartment (PVC) (Ahmed et al., 1997; Paris et al., 1997). The Arabidopsis genome encodes seven VSRs. All seven genes are expressed, and thus probably produce functional proteins (Chapter 2). Phylogenetic analysis indicated that all plant VSRs could be subdivided into three functional groups (Chapter 2). There are at least two *Arabidopsis* members represented in each group. Recent reports suggest that AtVSR1 is involved in the vacuolar trafficking of 2S albumin (Shimada et al., 2003). However, specific functions have not been assigned to any other AtVSRs. In this chapter, I describe the reverse genetic and biochemical approaches I used to determine the overlapping and unique functions of the AtVSRs. First, I took an antisense approach to get an overview of the function of the entire *AtVSR* family. Second, I used T-DNA insertion lines and RNAi to determine the functions of individual *AtVSR*s. Finally, to identify the VSR that functionally interacts with a known vacuolar protein, I used antibodies against known vacuolar proteins to coimmunoprecipitate AtVSRs. Once the procedure is optimized, the immunoprecipitated protein will be analyzed by proteomic techniques to identify the specific AtVSR that interacts with the vacuolar protein *in vivo*.

Materials and Methods

Plant Growth Conditions and Transformation Protocols

Arabidopsis (Columbia, unless otherwise indicated) seeds were sterilized in 50% bleach/0.05% Tween-20 (Sigma) and rinsed six times with sterile water. After cold treatment in 4°C for 24 hours, the seeds were sown on phytagar containing Murashige Minimal Organics Medium (Invitrogen/Gibco), and selection if needed, and grown under constant light conditions at approximately 23°C. After two weeks, the seedlings were transferred to a commercial soil mixture containing slow-release fertilizer pellets (Osmocote) and a fungicide (Marathon). The plants were grown under long day conditions (16 hours light/8 hours dark) at approximately 23°C.

Plant Transformation

The DNA constructs were transformed into *Agrobacterium tumefaciens* by a freeze-thaw protocol from Dr. Steve Farrand's lab. The GV3101 strain of Agrobacterium was grown in liquid media overnight at 28°C. Two milliliters of culture were transferred to an eppendorf tube on ice. The cells were washed in 10 mM Tris pH 7.5 and resuspended in cold luria broth. Two micrograms of plasmid DNA were added to the cells and the mixture was frozen in liquid nitrogen for five minutes. The mixture was transferred to 37°C for five minutes. Then, fresh luria broth was added and the cells were incubated in a 28°C shaker for three hours and transferred to selection agar. The plates were incubated at 28°C for two days. Antibiotic resistant colonies were screened for the presence of the construct by PCR and restriction digests. Then, the constructs were transformed into *Arabidopsis* using the floral dip protocol from Dr. Andrew Bent (Bent et al., 1994).

Photography and Microscopy

Seedlings were examined and photographed with a Leica MZIII Dissection Microscope with a Canon G4 digital camera attached. Microscopy of guard cells was accomplished on an Insight Confocal Microscope (Meridian) with a 20X and a 40X objective and micrographs were taken with a CCD camera (Hamamatsu).

Nucleic Acid Extraction from Plants and Subsequent Analysis

Genomic DNA was extracted from two small rosette leaves of an individual plant following a published protocol (Weigel and Glazebrook, 2002). PCR was carried out with Takara Ex-Taq (Takara Shuzo) following the manufacturer's instructions. Total RNA was extracted from rosette leaves using the RNeasy Plant Mini Kit (Qiagen). RT-PCR was carried out using 500ng total RNA, unless otherwise indicated, with the One-Step RT-PCR Kit (Qiagen) following the manufacturer's protocol.

Antisense AtVSR plants

The coding region for *AtVSR1/AtELP* was cloned into the PGA748 binary vector in the opposite orientation of a 35S promoter by Dr. Sharif Ahmed. The construct was transformed into *Arabidopsis*. To detect the presence of AtVSR protein, rosette leaves were excised from each plant and homogenized on ice in the presence of 3X Laemmli's buffer. The protein was separated by SDS-PAGE on a 10% polyacrylamide gel, blotted to nitrocellulose membrane, and probed with anti-VSR antibodies or anti-AtCPY antibodies followed by phosphatase-labelled anti-rabbit secondary antibodies. The blot

was developed in Tris buffer, pH 9.5 with Nitro Blue Tetrazolium (NBT) and Bovine Calf Intestinal Phosphatase (BCIP).

Characterization of the AtVSR3 gene trap line

The seeds were generously provided by Dr. Ueli Grossniklaus. Primers used in the genotyping PCRs were: *VSR3* forward – CCT TGT CCT TCG AAT TTG TTC TTT G; GUS Reverse – GCT CTA GAT CGG CGA ACT GAT CGT TAA AAC; *VSR3* Reverse – GTC CTT CCC GGG GAA TAA ATA GAT G. The PCR products were purified with a PCR purification kit (Qiagen) and sequenced by the Michigan State University DNA Sequencing Facility and the University of California, Riverside DNA Sequencing Facility.

Post-Transcriptional Silencing of the AtVSR3 Gene

The pFGC5941 dsRNA vector was a generous gift from Dr. Richard Jorgensen and Dr. Vicki Chandler. I followed the cloning strategy recommended by the ChromDB web page (http://ag.arizona.edu/chromatin/strategy.html). The primers to amplify the coding region for the last 10 amino acids and 3'UTR of *AtVSR3* were the following: Forward – TCT AGA GGC GCG CCT CCC GAA CCA CGT TGA ATG ATG AAC G; Reverse – GGA TCC ATT TAA ATA ACA ACA TGA ACT CTA AAA CAA GTA AC. The forward primer added an Xba I and Asc I restriction enzyme site (underlined) for cloning purposes. The reverse primer added a BamH1 and a Swa I restriction enzyme site (underlined) to the 3' end for cloning purposes. The PCR product was cloned into pGEM-T Easy (Promega) and sequenced by the UCR DNA Sequencing Facility. To add the 3' end of AtVSR3 in the forward orientation, this construct and the vector were

digested with AscI and SwaI. The relevant digestion products were purified from a 1% low melt agarose gel and the DNA was purified from the gel using a kit (Qiagen). The products were ligated with T4 Ligase (Roche) and transformed into DH5α. To add the same *AtVSR3* fragment in the opposite orientation, the new construct and the pGEM construct were digested with BamH1 and XbaI, ligated, and transformed as described above. The integrity of the final construct was confirmed by triple restriction digest with NotI, EcoRI, and PstI. The construct was transformed into *Arabidopsis*. Transgenic seedlings were obtained by germinating the seeds on selection media. RT-PCR was carried out for each putative transformant with primers corresponding to regions of the AtVSR3 gene outside of the domain used in the dsRNA construct: forward – CCT TGT CCT TCG AAT TTG TTC TTT G; reverse – TCT AGA GTC CTT CCC GGG GAA TAA ATA GAT G.

Germination Assay

Approximately 300 wildtype and mutant seeds were sterilized and transferred to Murashige Minimal Organics Medium (Gibco/Invitrogen) that contained either 3 μM ABA in methanol or 500 μl methanol as a control. The plates were stored at 4°C in the dark for three days. Then, the plates were transferred to room temperature conditions with constant light. Germinated and ungerminated seeds were counted for five days. Each seed was examined by a dissection microscope and a positive germination was considered to be a seed for which the radicle had emerged from the seed coat.

Analysis of Seedlings with Thermal Camera

Seedlings were grown as described for the germination assay without ABA for two weeks. The plates were photographed with a retail-grade digital camera and analyzed with a ThermaCAM 560 from FLIR Systems.

Reverse Genetics of AtVSR7

ET2539 seeds were generously provided by Dr. Rob Martienssen. The plants were genotyped by PCR with the following primers: *GUS* – GCT CTA GAT CGG CGA ACT GAT CGT TAA AAC; *AtVSR7* Forward – GAG ATG GGT TTA GTC AAC GGG AGA G. RT-PCR was carried out with the *AtVSR7* forward primer described above and the following primer: *AtVSR7* Reverse – GTA AAA GGC TCG GCT TCT GAT GGA AC. Western blot analysis of the AtCPY in mutant and wildtype plants was carried out as described in the Antisense materials and methods. SALK 005814 seeds were obtained from the Arabidopsis Biological Resource Center (ABRC). PCR genotyping was accomplished with the following primers: *VSR7*exon8forward – TAT CTA CTG GCT GCA AAT GTC CTG AAG GTT TCC, *VSR7*3'UTRreverse – CAA GAA GCT TTC ATA GCT CAG TTG GTT AG, SalkLBb1 – GCG TGG ACC GCT TGC AAC T.

Immunoprecipitation

Seeds were germinated and grown for seven days in liquid media as described above. Golgi were extracted from approximately 5g of whole seedlings following the protocol described in Munoz et al. (1996). Immunoprecipitations were carried out using a protocol from Dr. Alessandro Vitale's laboratory (D'Amico et al., 1992) with AtCPY antibodies or the corresponding preimmune antisera (Rojo et al., 2003a). The fractions

were separated by 10% SDS-PAGE, blotted to nitrocellulose membrane, and probed with anti-VSR antibodies (Ahmed et al., 1997).

Results

Antisense Silencing of the AtVSR Gene Family

The functions of the *AtVSR* gene family were examined by transforming *Arabidopsis* with a large, conserved region of the gene family in the antisense orientation relative to the constitutive 35S promoter. Expression of the transgene should result in post-transcriptional gene silencing (PTGS) of the endogenous gene family. A previous graduate student, Dr. Sharif Ahmed, attempted to express *antisense AtELP/AtVSR1* from the 35S constitutive promoter in *Arabidopsis* (Sharif Ahmed and Natasha Raikhel, unpublished results). Together, Dr. Ahmed and I were able to obtain only three transformants. The three plants set very few seeds and the germination rate of those seeds was very low.

I characterized progeny of the three transformants. Antibodies against the AtVSRs could not detect protein in three of the eight plants that germinated (Figure 3.1). The three plants that did not have detectable AtVSR protein also did not accumulate RT-PCR-detectable levels of mRNA for *AtVSR4*, *AtVSR6* and *AtVSR7* (data not shown). In addition, plant number three (Figure 3.1) did not accumulate *AtVSR1* transcript; plant number seven (Figure 3.1) did not accumulate *AtVSR5* transcript; and plant number eight (Figure 3.1) did not accumulate mRNA for *AtVSR2* or *AtVSR3* (data not shown).

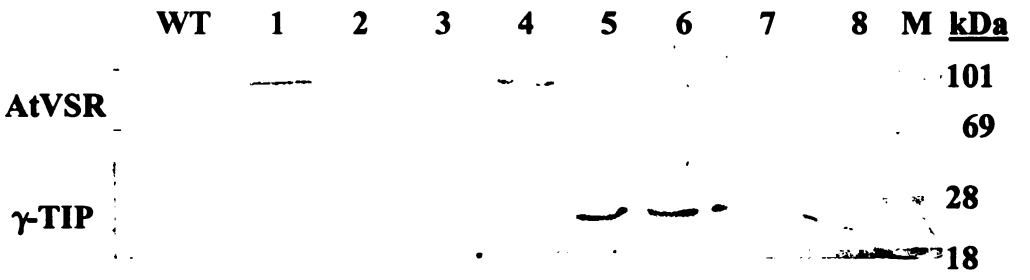

Figure 3.1. Some antisense AtVSR plants accumulate little or no AtVSR protein Protein was extracted from the leaves of eight antisense plants. The protein was separated by SDS-PAGE, blotted onto a nitrocellulose membrane, and probed with antibodies against the AtVSRs (Ahmed et al., 1997) or against the vegetative aquaporin, γ-TIP (28kDa). The γ-TIP antibodies are described in detail in Appendix C. Plants 3, 7, and 8 accumulated less AtVSR protein (~80kDa) than WT or other antisense plants.

The absence of a viable plant that silenced the entire *AtVSR* family indicated that a plant can not survive without some amount of VSR protein.

The three plants with reduced AtVSR family protein showed defects in gravitropism, leaf morphology, and flower morphology (Figure 3.2). Specifically, the roots of the seedlings consistently grew towards the plate lid (opposite the gravity vector) and pushed the cotyledons and leaves into the agar (Figure 3.2A). Furthermore, the stem of the bolting plant did not grow upward; it curled around inside the pot (compare Figures 3.2 C and D). The leaves formed "cups" and "umbrellas" or would roll under themselves (Figure 3.2B). There were also severe defects in flower development (Figure 3.2E). This phenotype was not observed in plants that accumulated wildtype levels or higher levels of AtVSR protein. Very few seeds were set by the antisense plants and again there was very low germination of the few seeds that were obtained. All of the progeny plants that germinated showed no suppression of *AtVSR* genes.

While it was clear that the reduction of AtVSR protein caused a pleiotropic phenotype, it was not obvious how protein trafficking to the vacuole was affected in the antisense plants. Therefore, I determined whether the vacuolar trafficking of AtCPY was affected in the antisense plants. AtCPY is an Arabidopsis soluble vacuolar protein that is often used as a marker for transport to the vacuole (Rojo et al., 2003b). The intermediate form of AtCPY is 43 kDa, and is processed to a 24 kDa protein after it is delivered to the vacuole (Rojo et al., 2003a). Therefore, the absence of the 24 kDa band would indicate that AtCPY vacuolar trafficking is blocked in the antisense plants. Two of these plants (3 and 8) lacked the 24 kDa form of CPY, indicating that at least one pathway to the vacuole is dependent on the expression of members of the *AtVSR* family (Figure 3.3).

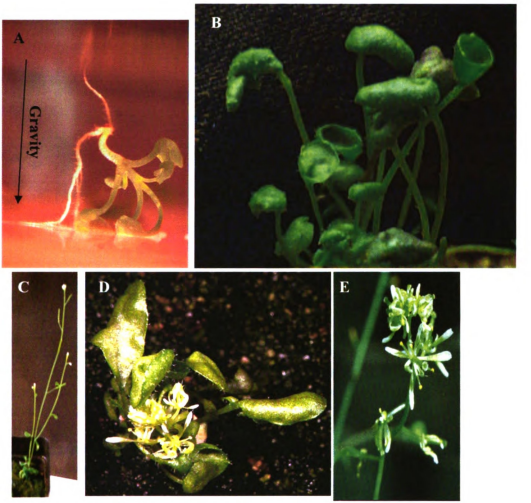

Figure 3.2. Antisense *AtVSR* plants showed defects in plant development. The seedlings showed defects in root and shoot gravitropism (A). The rosette leaves formed "cup" and "umbrella" shapes (B). While a wildtype plant bolts upward (C), the bolts of the antisense plants coil around the rosette (D). There are also severe defects in the flowers (E).

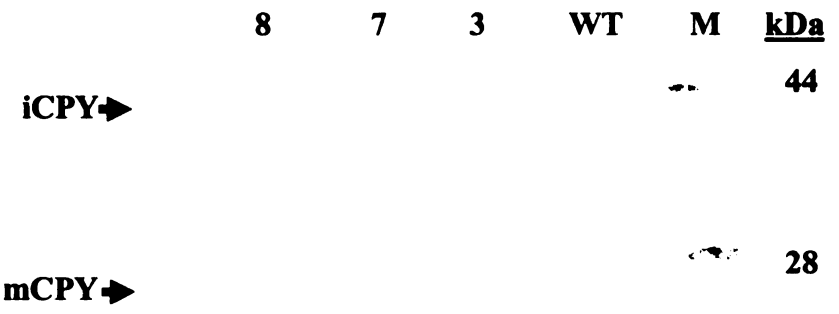

Figure 3.3. AtCPY is not delivered to the vacuole in two antisense plants. Vacuolar transport of AtCPY is determined by the processing of AtCPY from an intermediate form of ~43kDa to a mature 24kDa form. In wildtype plants, the intermediate form of CPY (iCPY) is delivered to the vacuole and processed to the mature form of CPY (mCPY; see WT lane). Under normal conditions, the transport and/or processing of AtCPY is slow enough that both forms are detected in wildtype by western blot analysis (Rojo et al., 2003). Thus, both forms of AtCPY are present in the lanes containing protein extracted from wildtype and sample 7, which accumulated a small amount of AtVSR protein. However, the mature form of AtCPY was not detected in protein extracted from plants 3 and 8, which did not accumulate any detectable AtVSR protein.

While it was not possible to obtain viable progeny from the *AtVSR* antisense plants and learn more from this approach, the results indicated that the *AtVSR* genes are important to many aspects of plant development. Furthermore, it is likely that at least some amount of AtVSR protein is essential to the plant since there was such a strong selection against the transmission of silencing to the progeny.

Functional Analysis of AtVSR3

RT-PCR and promoter::reporter fusion studies indicated that *AtVSR3* was expressed in guard cells and was regulated by ABA (Chapter 2). Similar results have been reported for other components of the endomembrane system, such as SYP121 and SYP61 (Leyman et al., 2000; Zhu et al., 2002). Therefore, I was very interested in determining whether AtVSR3 had a functional role in the ABA signal transduction pathway, and I took a reverse genetics approach to determine the function of *AtVSR3*. A line carrying an insertion in the *AtVSR3* gene was found in the Gene Trap collection from Dr. Rob Martienssen (Springer et al., 1995). The Gene Trap collection is a collection of *Arabidopsis* lines that were transformed with a non-autonomous transposon (Springer et al., 1995). TAIL-PCR was used to determine the location of each T-DNA in the collection and the information was stored in a BLAST-searchable database in Dr. Martienssen's laboratory. A BLAST search of this database with the coding region of *AtVSR3* revealed a gene trap line (GT3281) containing an insertion in the antisense orientation at 182 basepairs after the start codon of *AtVSR3* (Figure 3.4). I was unable to obtain homozygous mutants of this line, suggesting that the lack of functional AtVSR3 caused gametophytic, embryonic, or germination lethality.

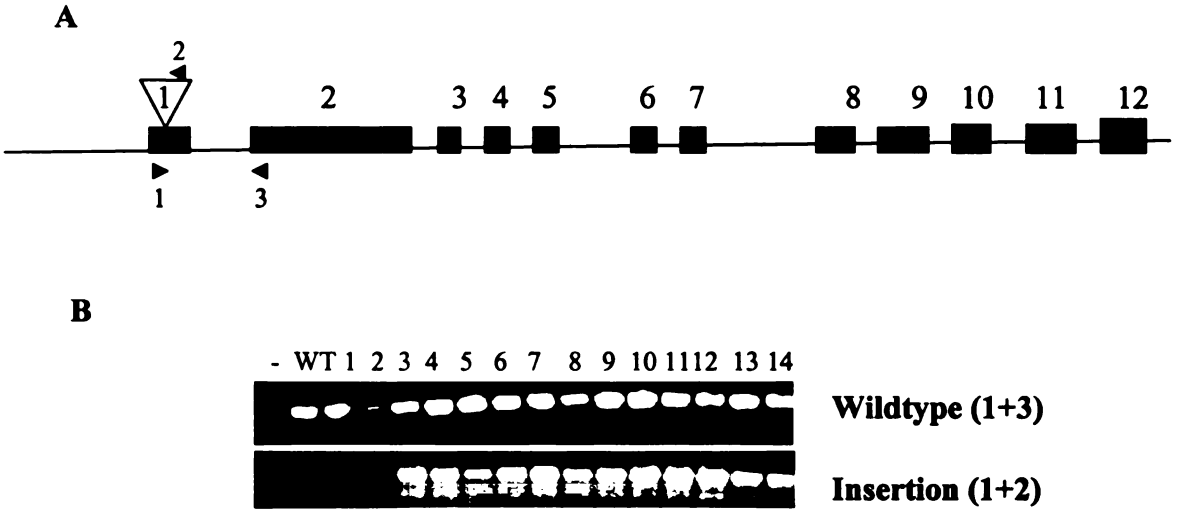

Figure 3.4. Gene trap insertion in the *AtVSR3* gene. The schematic depicts the *AtVSR3* gene with the number of each exon (black box) indicated above the exon (A). The insertion (white triangle) was in the first exon of the *AtVSR3* gene. Primers (black arowheads) were designed to genotype these plants. PCR genotyping of the plants indicated that I could not obtain a homozygous mutant (B). The top gel shows the products of PCR reactions using primers 1 and 3, demonstrating that all of the plants have at least one copy of the *AtVSR3* gene without an insertion. The bottom gel shows the products of PCRs using primers 1 and 2, demonstrating that one copy of the insertion is present in many of the plants.

I determined whether there was only one insertion in these plants by TAIL-PCR (Liu et al., 1995; Liu and Whittier, 1995). TAIL-PCR results for GT3281 indicated that at least two other insertions were present. This data, in conjuction with the potential lethal phenotype of the *AtVSR3* insertion, made GT3281 a difficult line to study.

Thus, I addressed the function of *AtVSR3* by using an RNAi approach to post-transcriptionally silence or at least reduce the accumulation of *AtVSR3* transcript. DNA encoding the last 10 amino acids of *AtVSR3* and the 3'UTR of *AtVSR3* were cloned into a RNA interference (RNAi) expression vector in which the *AtVSR3* DNA is in both the forward orientation and the reverse orientation and the two orientations are separated by a *GUS* intron (Chuang and Meyerowitz, 2000). The 3' end of *AtVSR3* was chosen because this region is not as well conserved among the *AtVSR* genes. Wildtype *Arabidopsis* plants were transformed with this construct. Seventy-one putative transformants were obtained from three independent transformations. RNA was extracted from each plant and 500ng of each sample was used as a template in semi-quantitative RT-PCR with *AtVSR3*-specific primers. An example is shown for fourteen plants in Figure 3.5A. The pixel intensity of each band was determined with the MCID Elite 6.0 image analysis software to objectively compare the amount of *VSR3* transcript accumulated in wildtype to that of the transformants (data not shown). The RNA extraction and RT-PCR experiments were repeated to ensure that the results were real. Three of the transformants accumulated reduced amounts of *AtVSR3* RNA and one transformant did not accumulate any *AtVSR3* transcript (Figure 3.5A). The seedlings that had little or no *AtVSR3* mRNA were small and accumulated more anthocyanins than wildtype seedlings and the true leaves showed slight defects in leaf shape (Figure 3.5B-D). An analysis of

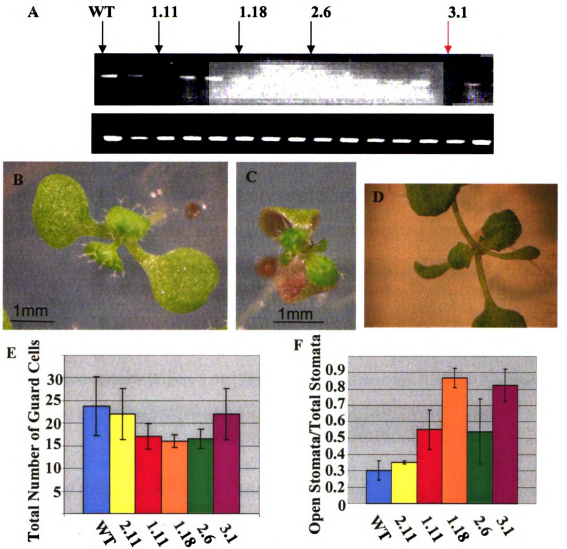

Figure 3.5. Visual characterization of *VSR3* RNAi plants. RT-PCR of wildtype (WT) and 14 putative transformants with *AtVSR3*-specific primers (A, top panel) or with *ubiquitin*-specific primers (A,bottom panel). Three transformants had reduced amounts of *AtVSR3* mRNA (A-1.11, 1.18, and 2.6) and one line that did not accumulate any *AtVSR3* mRNA (A-3.1), while the levels of *ubiquitin* were at normal levels. Seedling 3.1 is shown as a representative sample of the phenotype observed for 1.11, 1.18, 2.6, and 3.1 (C,D) compared to wildtype (B). The mutant seedlings were smaller than wildtype (B) with mishapen cotyledons that accumulated anthocyanins (C). The true leaves of the silenced lines had slightly aberrant leaf structure as shown in panels C and D. The abaxial surface of mature rosette leaves from four-week old plants produced similar amounts of guard cells as a wildype plant (WT) and a putative transformant (2.11) that accumulated normal levels of *AtVSR3* transcript (E). A closer look at the guard cells suggested that *AtVSR3* dsRNA plants did not have as many closed stomata as their wildtype counterparts under the same conditions (F). Closed stomata were considered to be those which had a diameter less than 2.5um.

the abaxial surface of mature rosette leaves revealed that the RNAi plants produced similar amounts of guard cells as their wildtype counterparts (Figure 3.5E). This suggested that AtVSR3 does not play a significant role in stomatal development. However, more guard cells in the mutants were open than in the wildtype plants under the same conditions (Figure 3.5F), suggesting that AtVSR3 may participate in guard cell opening and closing. If stomata of the mutants did not close, then the plants would have higher rates of transpiration, and thus remain cooler than wildtype plants (Merlot et al., 2002). Therefore, I compared the temperatures of wildtype and RNAi plants (line 3.1) for differences in temperature using a thermal camera (Figure 3.6). The analysis demonstrated that the mutants were an average 0.8°C ($P<0.0001$) cooler than their wildtype counterparts (Figure 3.6). These results indicated that AtVSR3 may play a role in stomatal movement.

The lack of VSR3 protein causes an ABA-insensitive phenotype in seeds

Guard cell movement is regulated by the phytohormone, ABA. In chapter two, I reported that *AtVSR3* expression was down-regulated by ABA. These results, combined with the phenotype of the RNAi lines suggested that AtVSR3 is part of the ABA signal transduction pathway. To confirm this, I asked whether AtVSR3 RNAi seeds were insensitive to ABA in a germination assay. Normally, ABA inhibits seed germination in wildtype seeds. However, mutants that do not respond to ABA will germinate in the presence of ABA. Wildtype and RNAi line 3.1 germination rates were compared for five days in the presence or absence of ABA (Figure 3.7). While wildtype seeds had a lower

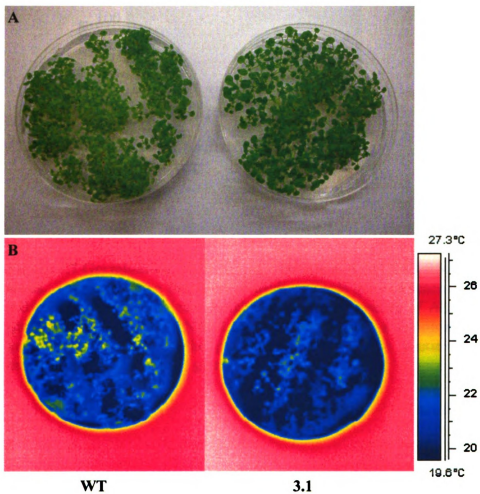

Figure 3.6. The average temperature of *AtVSR3* RNAi seedlings is less than the average temperature of wildtype seedlings. Wildtype and RNAi mutant line 3.1 seeds were sown on agar plates and grown for two weeks (A – wildtype on left and 3.1 on right. The plates were imaged with a thermal camera (B – wildtype on left and 3.1 on right) and the results were presented in a color format with a corresponding look up table. The temperatures of random spots on the plate were collected and compared between the wildtype and mutant plates. The average temperature over 33 random spots for two plates was 0.8°c higher in wildtype with a significance of $P<0.0001$.

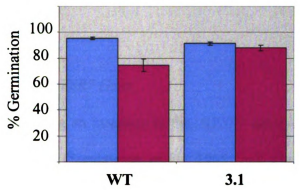

Figure 3.7 Germination frequency of wildtype and AtVSR3 RNAi seeds. The germination frequency of wildtype seeds in the absence of ABA (blue bar) is significantly higher than in the presence of ABA (purple bar). However, RNAi 3.1 seeds did not show a significant germination decrease in the presence of ABA. The y-axis is measuring the percentage of germination.

germination frequency in the presence of ABA, there was no significant difference between the germination rates of line 3.1 in the presence or absence of ABA (Figure 3.7). These results further indicated that AtVSR3 functions in the ABA signal transduction pathway.

Reverse Genetics of the AtVSR7 Gene

Two lines carrying an insertion in the *AtVSR7* gene were found in the enhancer trap collection (ET2539) (Sundaresan et al., 1995) and the SALK collection (SALK 005814) (Alonso et al., 2003). The enhancer trap line was found using the same techniques as described for finding the AtVSR3 gene trap line. I found the SALK insertion line by querying the TAIR database for information about *AtVSR7* (http://www.arabidopsis.org). The locations of the insertions for the SALK lines were also determined by TAIL-PCR (http://signal.salk.edu). I confirmed the location of each insert by TAIL-PCR. Both lines appeared to have a single insert based on TAIL-PCR and segregation analysis of the resistance gene (data not shown). The insertion in the enhancer trap line was located in the fourth intron of *AtVSR7*, while the insertion in the SALK line was located immediately after the stop codon of the gene. The homozygous plants did not produce mRNA of *AtVSR7* (Figure 3.8A). The plants had very stunted growth throughout their lifecycle (Figure 3.8B, C). This phenotype could be due to the production of less cells or due to the production of smaller cells. To address this issue, I compared the sizes of leaf epidermal cells (data not shown) and leaf mesophyll cells (Figure 3.8D) by microscopy of rosette leaves of the same age that had been cleared in

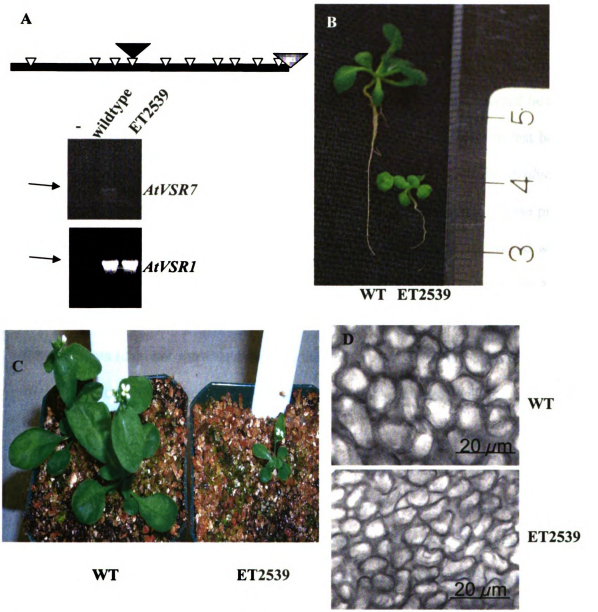

Figure 3.8. Characterization of *AtVSR7* knockout lines. A schematic of the *AtVSR7* gene is depicted in panel A. The exons are depicted as a black line whereas the introns are depicted as white triangles. The ET2539 insertion was located in the fourth intron (large, black triangle) and the Salk insertion was located immediately after the stop codon (large, grey triangle). Homozygous ET2539 plants do not accumulate *AtVSR7* mRNA, but do accumulate normal levels of other *AtVSRs* such as *AtVSR1* (A). The seedlings are very small (B), as were the mature plants (C). Micrographs of rosette leaf mesophyll cells are shown to demonstrate that the small plants had smaller cells (D, bottom panel) than their wildtype counterparts (D, top panel).

70% ethanol. The results indicated that the mutants produced smaller plants because their cells were smaller (Figure 3.8D).

Based on sequence homology, the putative function of AtVSR7 is a vacuolar sorting receptor. From this hypothesis, I predicted that an *atvsr7* mutant would be unable to deliver some proteins to the vacuole. This was a difficult hypothesis to test because very few soluble vacuolar proteins have been well characterized in *Arabidopsis*. However, antibodies for two Arabidopsis vacuolar markers are available. These proteins are AtAleurain and AtCPY (Ahmed et al., 2000; Rojo et al., 2003a). I checked whether the lack of AtVSR7 protein prevented the proper localization of AtAleurain or AtCPY. However, both proteins were delivered to the vacuole with the same efficiency as wildtype plants (data not shown), indicating that either AtVSR7 is not the VSR for these proteins or that another member of the AtVSR family can take over the function of AtVSR7 in its absence.

Immunoprecipitation of Vacuolar Sorting Receptors

A major challenge in determining the function of a plant VSR is that the cargo proteins for a specific VSR are not known. Thus, there is no straightforward assay that can be used to identify the *in vivo* function of each AtVSR. To address this issue, I took a biochemical approach to identify the specific AtVSRs that interact with a known vacuolar protein. Antibodies are available for characterized vacuolar proteins, such as AtCPY and AtAleurain. These antibodies could be tools to identify the functions of the AtVSRs. Seedlings were grown in liquid media for seven days in constant light and fractions enriched for Golgi were extracted from the tissue. The Golgi-enriched fraction

was solubilized in detergent and incubated with AtCPY antibodies. Then, the mixture was incubated with protein A sepharose beads, washed, and resuspended in Laemmli's buffer (Laemmli, 1970). Proteins from each of the steps were analyzed by western blot analysis with anti-VSR antibodies to determine whether a VSR was co-immunoprecipitated with the CPY antibody (Figure 3.9). An 80kDa band that reacted with anti-VSR antibodies was present in the AtCPY immunoprecipitation that was not present in the preimmune immunoprecipitation (Figure 3.9). A large amount of IgG heavy chain was also present, and this impeded the identification of the AtVSR by mass spectrometry techniques. I am now trying column chromatography to obtain a cleaner elution of the coimmunoprecipitation so that it will be possible to identify which member of the AtVSR family interacted with AtCPY in seven-day old seedlings.

Discussion

The overall function of the *AtVSR* gene family was studied by antisense technology. The germination rate of the transformants was very low and most of the seedlings did not suppress *AtVSR* expression. In chapter 2, I reported that many *AtVSRs* were expressed in seeds. In particular, *AtVSR1* expression was detected in seeds by RT-PCR, and promoter::GUS fusions, and seed ESTs for *AtVSR1* were identified. Furthermore, a knockout line of *AtVSR1* indicates that it may function in the vacuolar sorting of 2S Albumin in *Arabidopsis* seeds (Shimada et al., 2003). However, the knockout line itself is not lethal (Shimada et al., 2003). The low germination rate and low heritability of the antisense phenomena observed for the antisense plants in combination with the viable, null mutant of *AtVSR1* reported from Dr. Hara-Nishimura's

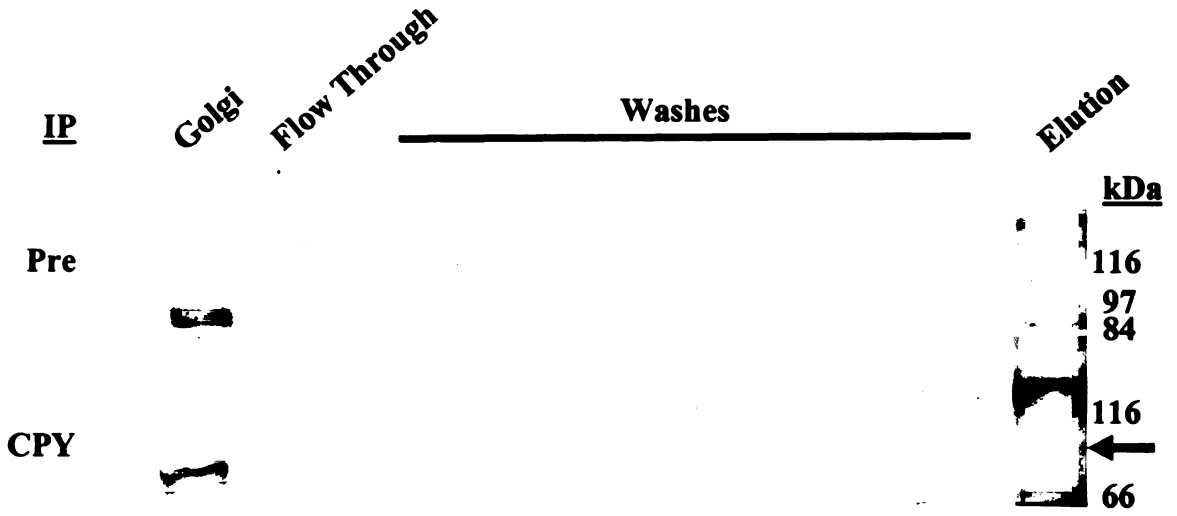

Figure 3.9 A VSR coimmunoprecipitated with AtCPY. Golgi-enriched fractions were immunoprecipitated (IP) with preimmune antibodies or anti-AtCPY antibodies. After three washes, the immunoprecipitations were resuspended in 3X Laemmli's buffer to release the proteins from the protein A sepharose beads. The protein from 40ul of each fraction was separated by SDS-PAGE and transferred to nitrocellulose membrane. The membranes were probed with antibodies against AtVSR protein. AtVSR protein (80 kDa) is present in the Golgi sample, the flow-through of the preimmune immunoprecipitation, and the elution of the CPY IP.

lab (Shimada et al., 2003) indicated that: *i*) another AtVSR has limited functional redundancy with AtVSR1, or *ii)* a different AtVSR serves an essential function in seeds.

The antisense VSR seedlings showed defects in shoot and root gravitropism. Shoot gravitropism is regulated by the plant hormone auxin and the endomembrane system is involved in auxin signal transduction (Kato et al., 2002; Surpin et al., 2003). Specifically, absence of functional AtVTI11 v-SNARE results in defects in shoot gravitropism (Kato et al., 2002; Surpin et al., 2003). Also, AtVTI11 colocalizes with AtVSRs (Zheng et al., 1999). The *vsr1* knockout mutant does not appear to have any obvious gravitropic defects (Shimada et al., 2003). Therefore, it is possible that at least one or more AtVSRs, other than AtVSR1, plays a role in gravitropism. However, this remains to be seen directly.

AtVSR3 plays a role in guard cell function

Results from the *AtVSR3* RNAi lines suggested that the little or no AtVSR3 protein results in plants that may have a decreased ability to close stomata relative to wildtype plants. I demonstrated that *AtVSR3* is ubiquitously expressed in seedlings and is only expressed in the guard cells of true leaves (Chapter 2). An ATP-binding cassette (ABC) transporter, *AtMRP5* (multidrug resistance-associated protein), has a very similar expression pattern by *promoter::GUS* fusions (Gaedeke et al., 2001). Furthermore, the guard cells of a knockout line for *AtMRP5* do not close in response to ABA (Klein et al., 2003). Therefore, *AtVSR3* and *AtMRP5* exhibited similar expression patterns and mutants that down regulate or lack the expression of these genes had similar phenotypes (Gaedeke et al., 2001; Klein et al., 2003). This suggests that the two proteins may

function in the same pathway. The MRP5 protein consists of 1501 amino acids. An NPIR motif (exactly N-P-I-R) occurs at amino acids 865-869, directly in the middle of the protein. The NPIR motif does function as a VSS from the internal sequence of the protein, ricin (Frigerio et al., 2001). However, MRP5 is an integral membrane protein and very little is known about the vacuolar trafficking of integral membrane proteins (Brandizzi et al., 2002). Clearly, the potential interactions of these two proteins should be examined further.

AtVSR7

The *vsr7* mutants were very small. *AtVSR7* expression was detected mostly in roots and at the distal ends of leaves under normal conditions (Chapter 2). *AtVSR7* is also upregulated in response to phosphate starvation (Hammond et al., 2003). Numerous vacuolar proteins are also upregulated in response to phosphate starvation (Hammond et al., 2003). Therefore, it is possible that AtVSR7 is the receptor for one or more of these precursor proteins.

Conclusions

The AtVSR family is thought to direct soluble VSS-containing proteins to the vacuole. I took reverse genetics and biochemical approaches to determine their specific functions. The overall results indicated that the AtVSRs play roles at multiple levels of plant development and are potentially involved in hormone-related signal transduction pathways.

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Chapter 4

Conclusions and Future Directions

Expression Analysis of the *AtVSR* Gene Family

The *AtVSR* gene family is composed of seven members. In this thesis, I present results showing the expression patterns of the *AtVSR* genes, as well as reverse genetics, and biochemical approaches to determine the functions of the *AtVSR* genes. The expression patterns of each gene indicated that the *AtVSRs* have tissue- and cell-type specific expression patterns. This result implies that the *AtVSR* genes are not completely redundant. Specifically, I found that none of the AtVSRs were expressed ubiquitously, which is the expression pattern of vacuolar sorting receptors in other eukaryotes. Also, one of the AtVSRs, AtVSR3, was specifically expressed in guard cells in an ABA-dependent manner. This result indicated that plants encode VSRs for specific functions in specific signal transduction pathways. This has not been reported for any other eukaryotes, and thus is an important scientific contribution.

Subcellular Localization of AtVSRs

Homologues of the AtVSRs in other plants have been localized to organelles outside of the CCV pathway, such as ER bodies and PAC vesicles. With this in mind, studies of the AtVSRs should be extended to the subcellular localization of each protein. This can be accomplished by transforming *Arabidopsis* plants with tagged-versions of the individual *AtVSR* genes. Towards this end, we have transformed *Arabidopsis* with a construct that encodes the *AtVSR1* promoter and the *AtVSR1* gene fused to the coding region of *YFP* (C. Sambojou and N. Raikhel, unpublished data). A similar construct for *AtVSR3* has also been transformed into *Arabidopsis*. The YFP marker can be used to determine the subcellular localization of an individual AtVSR by microscopy and

biochemical techniques. Confocal microscopy would give some indication of the subcellular localization of the AtVSRs. However, immuno-electron microscopy with anti-YFP antibodies would give a higher resolution image, and thus a more conclusive localization of the protein. It should also be possible to fractionate the organelles by sucrose density gradient and look for the YFP protein by western blot analysis with anti-YFP antibodies. A comparison of the YFP fractionation pattern with markers for other endomembrane organelles and vesicles would determine the localization of the chimeric protein. These experiments will determine where each AtVSR participates in vacuolar protein targeting.

Recycling of Plant VSRs

Regardless of an individual VSR's position in the vacuolar targeting pathway, current models predict that all VSRs are recycled from the recipient compartment to the donor compartment. While some aspects of the recycling mechanism have been pursued, the actual process has not been demonstrated in plants (Sanderfoot et al., 1998; Happel et al., 2004). The plant AtVSRs, yeast Vps10, and mammalian MPR all have a tyrosine motif in the cytoplasmic tail that interacts with AP-1 for clathrin-coat formation around the budding vesicle (Sanderfoot et al., 1998; Bonifacino and Dell'Angelica, 1999; Deloche et al., 2001) In addition to AP-1, a family of sorting nexin (SNX) proteins may also share the responsibility of receptor protein localization (Kurten et al., 1996). A yeast 2-hybrid assay using the C-terminus of the epidermal growth factor receptor (EGFR) identified a sorting nexin protein (SNX1) that interacted with the C-terminus of EGFR (Kurten et al., 1996). A YLVI motif in the C-terminus of EGFR is necessary for the

interaction between EGFR and SNX1 (Kurten et al., 1996). SNX1 is a hydrophilic peripheral membrane protein that contains a p40 phox (PX) domain that binds phosphotidylinositol 3-phosphate (Ponting, 1996). The C-termini of SNX1 has a coiled-coil domain that is important for dimerization between SNX proteins (Seaman and Williams, 2002). SNXs have been shown to interact with various receptors that are transported through the endomembrane system (Haft et al., 1998).

Three proteins in yeast, Mvp1p, Vps5p, and Vps17p, share significant homology with human SNX1 (Horazdovsky et al., 1997). Mvp1p also shares significant homology to dynamin and thus may perform a function in vacuolar sorting that is distinct from SNX1. However, there is evidence that Vps5p and Vps17p function together to perform a role in receptor recycling that is orthologous to SNX1 (Horazdovsky et al., 1997). *vps5p* and *vps17p* mutants accumulate numerous small vacuoles and secrete CPY. The defect in CPY sorting to the vacuole is due to the mislocalization of the CPY sorting receptor, Vps10. Vps5p and Vps17p dimerize through their coiled-coil domains to form part of the retromer complex in yeast. The retromer complex also consists of Vps26p, Vps29p, and Vps35p. Together, this complex is thought to mediate the recycling of receptor proteins within the endomembrane system. Human SNX1 was shown to bind a homolog of yeast Vps27p. Therefore, Vps27p may also be a member of the retromer complex.

A retromer complex has not yet been characterized in plants. However, there are genes in the *Arabidopsis* genome whose deduced amino acid sequences share significant sequence homology with human SNX1 and yeast Vps5p. Likewise, the C-termini of AtVSRs also have YLVI motifs. Therefore, *Arabidopsis* may also utilize a retromer

complex to recycle vacuolar sorting receptors from the prevacuolar compartment to the TGN. This can be further examined in two ways. First, immunoprecipitation of AtVSRs with generic previously characterized AtVSR antibodies (Ahmed et al., 1997; Li et al., 2002) should pull out cytosolic interacting factors that can be analyzed by protein blots and/or mass spectrometry. Second, the localization of AtVSRs should be determined in knockout mutants for the Arabidopsis homologues of the retromer complex. These two approaches should determine whether the mechanism of VSR recycling is analogous to yeast or mammalian systems.

Reverse Genetics Approaches to Determine the Functions of AtVSRs

The functions of the AtVSRs were examined by reverse genetic techniques such as post-transcriptional gene silencing and the characterization of T-DNA insertion lines. By attempting to silence the entire *AtVSR* family, we obtained plants that had very low germination rates, defects in root and shoot gravitropism, as well as defects in leaf and flower morphology. These processes are regulated by hormones such as ABA, gibberellic acid and auxin. With this in mind, the *promoter::GUS* fusion lines for the *AtVSR* promoters and RT-PCR should be used to determine if any of the *AtVSR* genes are upregulated or down-regulated in response to phytohormones. For example, the plants expressing the *promoter::GUS* constructs could be treated with phytohormones or subjected to different environmental conditions and subsequently stained to determine GUS activity. Similar research was accomplished in the studies of auxin-responsive genes such as DR5 (Sabatini et al., 1999). The results of these experiments would lead to hypotheses that could be tested in knockout lines of the *AtVSRs*.

Reverse Genetics to Understand the Function of AtVSR3

Examination of RNAi plants that partially or completely silenced *AtVSR3* revealed that the reduction or lack of AtVSR3 causes the stomata to remain open in greater numbers than stomata from wildtype plants. The temperature of these plants was lower than the temperature of wildtype plants as a result of the non-responsive stomata. Similar results were observed for the ABC transporter, AtMRP5 (Klein et al., 2003), suggesting that AtMRP5 and AtVSR3 function in the same pathway. Surprisingly, AtMRP5 has an NPIR motif. An exciting speculation is that AtVSR3 is a vacuolar sorting receptor for AtMRP5. Many conventional strategies to address this issue will be difficult because both proteins are integral membrane proteins. We already have putative transformants of *AtVSR3* fused to *YFP*. Therefore, we can prepare a construct containing *AtMRP5* fused to *CFP* and transform it into the *AtVSR3:YFP* plants. The leaves of the double-transgene plants can be tested for fluorescence energy resonance transfer (FRET) activity by confocal microscopy (Huang et al., 2001; Shah et al., 2002). The YFP fluorescence is distinguished from the CFP fluorescence by the presence of specific emission filters in the light path. Another possibility is to make antibodies against MRP5 for use in colocalization experiments with AtVSR3.

Identification of the In Vivo Targets of AtVSRs

In order to fully characterize an AtVSR, we need to identify the putative *in vivo* targets of the AtVSRs. The best method for accomplishing this is to transform *Arabidopsis* with epitope-tagged versions of the AtVSRs. In this way, an individual AtVSR can be specifically immunoprecipitated. The proteins that coimmunoprecipitate

with the tagged protein can be analyzed by western blot or by mass spectrometry to determine their identities. This will determine whether each AtVSR interacts with a specific type of VSS and whether each interaction is regulated by pH, calcium, or by another mechanism. The approaches described above will lead to a more comprehensive understanding of VSR-mediated trafficking in plants. An added benefit to this approach is that we will likely pull out proteins that interact with the cytoplasmic tail of the AtVSR as well. The identification of these proteins will be useful in determining the subcellular localization and recycling of the AtVSRs, a question that was discussed earlier in this chapter.

Conclusions

Studying the *AtVSR* gene family presents an excellent opportunity to understand how plants differ from higher eukaryotes. The results presented in this thesis demonstrate that the endomembrane system of plants follows the same paradigm that has been described in other eukaryotes. However, the diversity of the plant endomembrane system has been expanded to accommodate the unique lifestyle of plants. Our lab is now using high throughput and systems biology approaches to describe these processes in more detail.

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APPENDICES

APPENDIX A

Supplementary Information for Chapter 2

Table A.1. ESTs and cDNAs for the *AtVSR1* Gene

Source Tissue and Number of ESTs and/or cDNAs Found	GenBank Accession(s)
Developing Seeds 5-13 daf (1)	BE529517
Benning Immature Seed cDNA Library (1)	M74D05
Green Siliques (4)	AV567436; Z35038; Z35039; Z38123
3-Day Old Seedling Hypocotyls (1)	AA042124
Rosette Plants Subject to Dehydration (5)	AV794541; AV826147; AV793751; AU238557; AV794045
A-PRL2 (4)	AA650957; R30384; AA605487; R90202

Table A.2. Promoter Elements Found in the *AtVSR1* Promoter

Promoter Element (Number of Occurences in promoter) Location(s) relative to the start codon	DNA Sequence	Description	PLACE Accession	Reference
AACA CORE[a] -1450 to -1200	AACAAAC	Endosperm expression	S000353	Wu et al., 2000
-300 Element (1) -10	TGTAAAGG	Found in the promoters of seed storage genes	S000122	Thomas and Flavall, 1990
TATCCA[b] (4) -1450, -650, -350, -250	TATCCA	GA and Sugar Response Element found in amylase promoters	S000403	Lu et al., 2002
DPBF Consensus Motif (2) -600, -400	ACACNNG	Embryo Expression and/or ABA Response	S000292	Kim et al., 1997
E Box (10) -1570, -1550, -1450, -950, -760, -740, -730, -550, -480, -410	CANNTG	Embryo Expression and/or ABA Response	S000144	Kim et al., 1997
SEF4 (2) -1300, -150	RTTTTTR	Enhances expression in embryo cotyledons	S000103	Lessard et al., 1991; Fujiwara and Beachy, 1994
SEF3 (1) -1600	AACCCA	Enhances expression in embryo cotyledons	S000115	Lessard et al., 1991; Fujiwara and Beachy, 1994
RY Repeat (1) -700	CATGCAT[c]	seed and leaf expression	S000100	Fujiwara and Beachy, 1994
Root Motif (5) -1500, -1350, -1175, -990, -800	ATATT	Root expression	S000098	Ehmayan and Tepfer, 1995

[a] Signal Scan identified the AACA motif. Wu, et al (2000), reported that the AACA motif only functions in combination with a GCN4 motif and an ACGT motif in close proximity. This, in fact, occurs for the *AtVSR1* promoter.

[b] Signal Scan identified the TATCCA motif. Lu, et al. (Lu et al.) reported that when the TATCCA motif is in close proximity to the TAACAAA motif, the gene is strongly expressed in response to Giberellic Acid (GA). The -1450bp TATCCA element is 150 bases away from a TAACAAA motif, suggesting that this region may function as a GA-response element.

[c] In promoter::reporter fusions with regions of the promoter for the gene encoding the α' subunit of the Soybean β-conglycinin protein, the presence of the CATGCAT version of the RY element with the SEF3 and SEF4 motifs resulted in GUS activity in the seeds as well as in the leaves of transgenic plants. However, the presence of the CATGCAC version of the RY element with the SEF3 and SEF4 motifs resulted in repression of *GUS* in leaves while *GUS* expression in seeds was unaffected.

Table A.3. Promoter Elements That Are Present in the *AtVSR2* Promoter

Promoter Element (Number of Occurences in promoter) Location within the *AtVSR2* Promoter	**DNA Sequence**	**Description**	**PLACE Accession**	**Reference**
Root Motif (26) -2500, -2450, -2300, -2200, -2025, -1850, -1800, -1525, -1500, -1400, -1300, -1250, -1225, -1000, -950, -750, -725, -650, -600, -475, -400	ATATT	Root expression	S000098	Ehmayan and Tepfer, 1995
AACA CORE[a] (2) -501, -325	AACAAAC	Endosperm expression	S000353	Wu et al., 2000
SEF4 Motif(12)[b] -2900, -2825, -2325, -2300, -2125, -2100, -1900, -1175, -200, -175, -150, -140	RTTTTTR	Enhances expression in embryo cotyledons	S000103	Lessard et al., 1991
SEF3 Motif (1)[b] -2000	AACCCA	Enhances expression in embryo cotyledons	S000115	Fujiwara and Beachy, 1994
RY Element (1)[b] -600	CATGCAA	Enhances expression in embryo cotyledons	S000100	Fujiwara and Beachy, 1994
DPBFCOREDCDC3 (2) -800, -1550	ACACNNG	Embryo Expression and/or ABA Response	S000292	Kim et al., 1997
E-Box (8) -2400, -1700, -1450, -1000, -850, -800, -700, -350	CANNTG	Embryo Expression and/or ABA Response	S000144	Kim et al., 1997
TGACGT Motif (1) -1600	TGACGTAA	Expression in cotyledons of germinated seeds	S000377	Yamauchi, 2001

[a] Signal Scan identified the AACA motif. Wu, et al (Wu et al.), reported that the AACA motif only functions in combination with a GCN4 motif and an ACGT motif in close proximity. This combination also occurs for the *AtVSR2* promoter with ACGT elements occurring at -510 and -601 as well as a GCN4 element occurring at -551.

[b] The combination of the SEF4 element, the SEF3 element, and the RY element cause the enhancement of expression of seeds, specific the cotyledons (Fujiwara and Beachy, 1994). In promoter::reporter fusions with regions of the promoter for the gene encoding the α' subunit of the Soybean β-conglycinin protein, the presence of the CATGCAT version of the RY element with the SEF3 and SEF4 motifs resulted in GUS activity in the seeds as well as in the leaves of transgenic plants. However, the presence of the CATGCAC version of the RY element with the SEF3 and SEF4 motifs resulted in repression of *GUS* in leaves while *GUS* expression in seeds was unaffected.

Table A.4. Promoter Elements That Are Present in the *AtVSR3* Promoter

Promoter Element (Number of Occurences in *AtVSR3* Promoter) Location(s) within the *AtVSR3* Promoter	**DNA Sequence**	**Description**	**PLACE Accession**	**Reference**
DOF Core Motif (25) -25, -50, -100, -325, -350, -375, -450, -525, -585, -590, -600, -700, -725, -750, -850, -895, -900, -935, -950, -1100, -1125, -1150, -1525, -1550, -1575	TAAAG	Guard Cell Specific Expression	S000387	Plesch et al., 2001
DRE1 Core Motif (1) -1400	ACCGAGA	ABA and/or late embryogenesis; May also act as a repression element.	S000401	Kizis and Pages, 2002

Table A.5. Promoter Elements in the *AtVSR4* Promoter

Promoter Element (Number of Occurrences within the *AtVSR4* Promoter) Location within the *AtVSR4* Promoter	**DNA Sequence**	**Description**	**PLACE Accession**	**Reference**
AACA Core (1) -190	AACAAAC	Endosperm expression	S000353	(Wu et al., 2000)
SEF4 (3) -300, -450, -1360	RTTTTTR	Enhances expression in embryo cotyledons	S000103	(Lessard et al., 1991)
SEF3 (2) -530, -1340	AACCCA	Enhances expression in embryo cotyledons	S000115	(Lessard et al., 1991)

Table A.6. Promoter Elements in the *AtVSR6* Promoter

Promoter Element (Number of Occurrences) Location(s) within the *AtVSR6* Promoter	**DNA Sequence**	**Description**	**PLACE Accession**	**Reference**
Pyrimidine Box[a] (1) -450	CCTTTT	GA Response and sugar starvation response	S000259	Mena et al., 2002
TAACAAA Box (2)[a] -526, -1110	TAACAAA	GA Response and sugar starvation response	S000020	Mena et al., 2002
TATCCA Box (1)[a] -700	TATCCA	GA Response and sugar starvation response	S000403	Mena et al., 2002
CGACG Box (1)	CGACG	Expression during sugar starvation	S000205	Hwang et al., 1998

[a]The promoters of GA-induced genes usually have a tripartite motif consisting of the TAACAAA box, the pyrimidine box, and the TATCCA box (Mena et al., 2002).

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Appendix B

Avila, E.L*., Zouhar, J*., Agee, A.E., Carter, D.G., Chary, S.N., Raikhel, N.V. (2003). Tools to Study Plant Organelle Biogenesis. Point Mutation Lines with Disrupted Vacuoles and High-Speed Confocal Screening of Green Fluorescent Protein-Tagged Organelles. Plant Physiology, 133(4): 1673-1676.

***E.L. Avila and J. Zouhar contributed equally to this work.**

We have focused our studies in the past several years on understanding protein trafficking from the secretory system to the vacuole—an organelle present in all plant cells. Here, we report an approach for generating and screening plants with defects in vacuolar biogenesis. Plant vacuoles are commonly known to be multifunctional organelles, and recent findings have even demonstrated a variety of new roles for vacuoles and the vesicles that deliver cargo to them. Although it has always been assumed that vacuoles are essential for plant survival, the recent isolation of a T-DNA-tagged mutant called *vcl1 (vacuoleless1)* has unequivocally demonstrated that vacuoles are vital organelles (Rojo et al., 2001). Mutations in the yeast (*Saccharomyces cerevisiae*) ortholog of VCL1, VPS16, also block vacuole biogenesis and affect all known vacuolar protein transport pathways in yeast; however, in contrast to VCL1, the VPS16 gene product is not essential (Horazdovsky and Emr, 1993). A major effect of VCL1 inactivation is that it blocks the formation of vacuoles, leading to embryonic lethality. Thus, although the isolation of *vcl1* served to emphasize the importance of plant vacuoles to plant growth and development, it is difficult to gain additional information about vacuole biogenesis from an embryo lethal mutant. Some important proteins that likely mediate trafficking to the vacuole are represented by single genes in the *Arabidopsis* genome. For example, each of the six members of the AtC-VPS complex for which VACUOLELESS1 is a member is encoded by a single gene (Rojo et al., 2003); thus, null mutations in these genes would also most likely be lethal.

Similar conclusions were drawn when several knockout mutants from the SNARE family were isolated. Although a T-DNA insertion into the SYP61/OSM1 syntaxin is viable (Zhu et al., 2002), some reported null mutations of syntaxin genes are not tolerated

by the plant. For example, a T-DNA disruption of a single member of the SYP2 and SYP4 gene families is gametophytic lethal (Sanderfoot et al., 2001). Another knockout mutant, *"knolle" (syp111),* is embryo/seedling lethal (Lukowitz et al., 1996). However, a point mutation in the SYP22/SGR3 gene is viable, and the mutant lacks the shoot gravitropic response (Yano et al., 2003). Thus, it becomes apparent that the isolation of plants with point mutations would be a very valuable tool to isolate viable mutants for studying plant vacuolar biogenesis. With this in mind, we wanted to identify mutants with small defects in vacuolar biogenesis genes that at the same time would not be lethal to the plant.

To allow for effective visualization of vacuolar structure, we chose *Arabidopsis* lines expressing a fluorescent tonoplast marker, green fluorescent protein (GFP): Δ-tonoplast intrinsic protein (TIP), under the control of the 35S promoter (Cutler et al., 2000). The tonoplast-localized GFP fusion protein in the tonoplast of these plants is easily visualized by confocal microscopy (Fig. B.1, A–D). Homozygous seeds from 35S::GFP: Δ-TIP plants were obtained, and vacuoles from these plants were isolated using the technique described previously by Ahmed et al. (Ahmed et al.). Proper GFP:Δ-TIP localization at the tonoplast was confirmed by microscopy (Fig. B.1E). Seeds from homozygous plants were then treated with ethyl methanesulfonate (EMS) to induce 1-bp changes throughout the genome of this line of plants. The Meridian Insight Point Confocal Microscope (Meridian, Okemos, MI) was used to screen 7-d-old seedlings from the M2 generation for broken or malformed vacuoles, mistargeting of the GFP:Δ-TIP chimeric protein, or other interesting phenotypes. The Meridian Insight Confocal has real-time ocular viewing confocal capability that allowed us to rapidly screen large

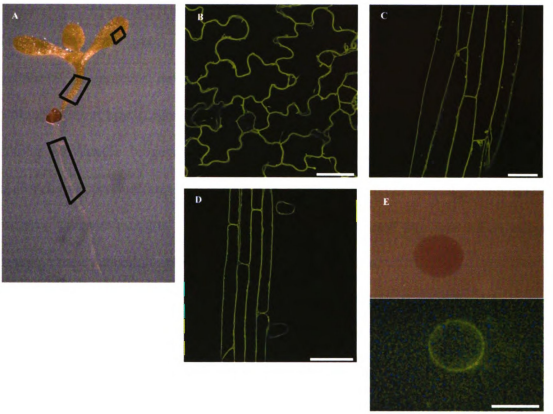


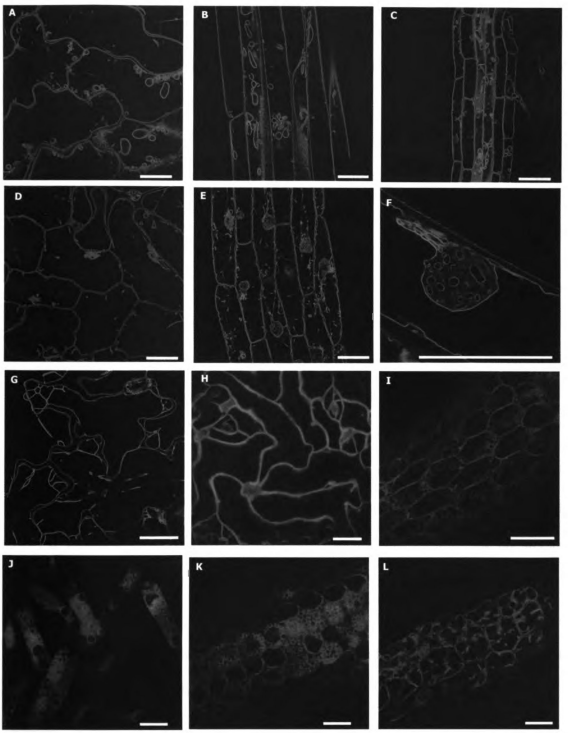
Figure B.1 GFP:-TIP is expressed in the tonoplast of 35S::GFP: -TIP transgenic seedlings (A) such as cotyledon epidermal cells (B), hypocotyls (C), and roots (D). E, Example of an isolated vacuole stained with neutral red (top) and with GFP fluorescence (bottom). Scale bar 40μ.

numbers of seedlings. For each seedling, three types of tissues were examined: cotyledons, hypocotyls, and roots (Fig. B.1A).

Thus far, we have screened 9,175 M2 EMS seedlings (56 pools; approximately 160 seedlings per pool) using confocal microscopy. Seedlings (620; 7%) showed mutations in pigment development, indicating that mutagenesis and the screening protocol were robust (Lightner and Caspar, 1998). Originally, 211 putative mutants with defects in vacuole biogenesis were obtained; however, 110 died before setting seed and/or did not produce any seed (Fig. B.2, I–L), resulting in our current population of 101 putative vacuolar mutants. These mutants have been sorted into four broad categories based on their subcellular phenotypes in the M2 generation.

The first category of mutants (*bub [bubble-bath]*) is characterized by increased numbers of small vacuolar vesicles in the cell (Fig. B.2, A–C). Forty-six plants fell into this category. Among these plants, the *bub* phenotype was observed either in roots (three plants), in cotyledons (29 plants), or in hypocotyls and cotyledons (six plants). Although *bub* plants appear to have a large, central vacuole, they also have increased numbers of vesicles decorated with GFP:Δ-TIP relative to the parental line. The severity of the phenotype correlated with plant lethality because plants with the highly pronounced *bub* phenotype did not survive (Fig. B.2, I–K) or did not produce seeds. The second category of mutants contained large aggregates of GFP fluorescence (*agg*; Fig. B.2, D–F). Thirty-four plants fit into this category. Among these plants, the *agg* phenotype was observed either in roots (14 plants), in hypocotyls (nine plants), in cotyledons (one plant), in hypocotyl and cotyledons (one plant), or in hypocotyls and roots (one plant). Upon closer examination of M3 *agg* plants, we observed that some of the aggregates are

Figure B.2. Examples of vacuolar mutants identified. *bub* mutants have increased numbers of vesicles in the cotyledons (A), hypocotyls (B), or roots (C). Examples of *agg* mutants with aggregates in the cotyledons (D) and hypocotyls (E). A closer look at the aggregates (F) reveals a membrane-bound vesicular structure. G, Example of the *tvs* mutant class with increased transvacuolar strands. Some of the mutants had complex phenotypes, such as disruption of cell shape (H). I to L, Many interesting M2 vacuolar mutants that did not survive to the M3 generation. Images A to G were collected by a Leica TCS SP2/UV Confocal Microscope (Leica Microsystems, Wetzlar, Germany). Images H to L were collected on a Meridian Insight Point Confocal Microscope with a CCD-cooled camera. Scale bar 40 um.


A
B
C
D
E
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L

membrane bound compartments containing clusters of vesicles (Fig. B.2F). The third category contained mutants that showed vacuoles apparently transected by transvacuolar strands (*tvs*; eight plants; Fig. B.2, G and L). Among these plants, the *tvs* phenotype was observed either in roots (three plants), in cotyledons (two plants), in hypocotyl (one plant), in hypocotyl and cotyledons (one plant), or in hypocotyls and roots (one plant). Further investigation of the M3 generation of *tvs* plants revealed that their vacuoles were extremely dynamic with the continuous rearrangement of the transvacuolar strands. Additional mutants appear to have unique and more complex phenotypes (Fig. B.2H). Thirteen viable M2 plants were clustered into this category. The unique phenotypes included defects in the regular pattern of the cotyledon epidermal cells (seven plants). Interestingly, approximately one-half of seedling-lethal mutants (60 plants; Fig. B.2, I–L) showed complex or unique phenotypes throughout the seedling.

Because genetic mapping of these mutants will involve large numbers of F2 seedlings, we are working toward developing a high-throughput screening process. We are now using an Atto Pathway HT high-throughput confocal microscope system (Atto Bioscience, Rockville, MD) with culture plates of our own design for germinating and growing seedlings (Fig. B.3). All tissues are screened manually or by an advanced automated imaging routine without damaging the seedlings (Fig. B.3, C and D). An example of the wild-type and mutant (*bub*) images produced by the Atto Pathway microscope can be accessed in supplemental data, available in the online version of this article at http://www.plantphysiol.org.

Conclusions

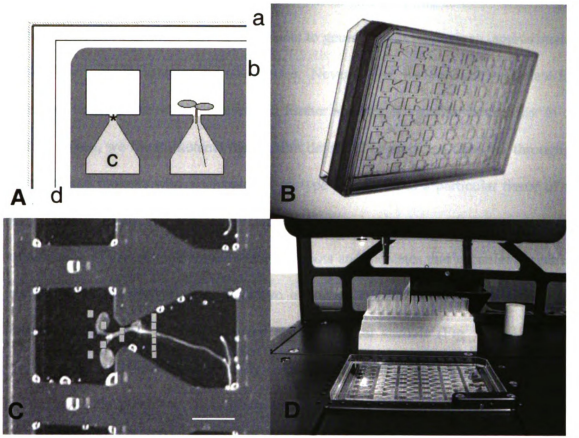

Figure B.3. A, Assembly of screening plates: a standard multiwell plate lid (A) has a silicone gasket with 48 hourglass-shaped holes (B) applied to its outer surface. The bottom half of each opening (C) is filled with solid growth medium, and a seed is pipetted into the neck. A sheet of cellophane (D) flattens and seals the screening wells while allowing gas exchange. The cellophane is replaced with a coverslip for imaging. Populated plates are stacked together, sealed with surgical tape, and then incubated in a vertical position for 7 d. To keep the seeds hydrated, a layer of agar is deposited on the inside of each plate before it is populated and stacked. B, Populated plates viewed from above. Gravitropism assures vertical orientation of seedlings, which are illuminated evenly from all sides. C, Seedling in single well. The squares indicate the standard 5 2 6 search pattern for automatically finding cotyledon, hypocotyl, and root, respectively. Scale bar 3 mm. D, Plate in the climate controlled imaging chamber of the Pathway HT automated imager. All tissues are close enough to the cover glass to be imaged by the UApo/340UV 20/0.75NA objective lens, which moves on linear motors below the sample. For more information on microscopy methods described above, visit http://www.cepceb.ucr.edu.

Using a mutagenized transgenic line expressing a tonoplast-localized protein fused to GFP, we were able to screen for vacuolar biogenesis mutants using confocal microscopy. Although we used EMS to mutagenize plants to generate point mutations, approximately 50% of the vacuolar mutants did not survive. Nevertheless, we were able to isolate four groups of mutants that would be useful in further analysis of vacuolar biogenesis. In our mutant screen, we found mutants that exhibit defective or modified vacuoles throughout the plant and mutants whose vacuolar phenotype is specific to a particular tissue of the plant. This suggests that the endomembrane system in shoots can be uncoupled from organization of the endomembrane system in roots and indicates that vacuolar biogenesis has tissue-specific components. It is also important to note that we never recovered a mutant seedling that completely lacked a large, central vacuole (Fig. B.2, A–L). This result supports previous conclusions by Rojo et al. (2001) that the vacuole is an essential organelle to the plant cell. It is likely that an approach encompassing only transient disruptions of vacuolar biogenesis components, such as chemical genetics, will be beneficial to directly target many fundamental vacuolar biogenesis proteins.

Although the screen was originally performed using the Meridian Insight Point Confocal Microscope, a system has been developed that grows up to 48 seedlings on a multiwell plate lid, which can be imaged automatically. Although fully automating the process is at an early stage of development, it will eventually increase the scope of possible experiments, including chemical genetics screens to test for the effects of drugs on seedling germination and tissue development. One of the challenges will be to manage the large volume of data generated by automated screening. Similar screening approaches could offer an excellent opportunity to study the biogenesis of other plant organelles.

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Appendix C

Identification of the Protein Contents of Plant Cell Vacuoles

Introduction

One possible distinctive feature of AtVSR proteins may be that each interacts with a different type of VSS, which in turn may target the protein to a distinct vacuole (Vitale and Raikhel, 1999). To fully explore this possibility, it is necessary to characterize many different soluble vacuolar proteins to use as markers. A proteomics approach was used to identify protein markers for specific types of vacuoles. I attempted to collect distinct types of vacuoles by Fluorescence-Activated Cell Sorting (FACS) and analyze their protein contents.

Materials and Methods

Preparation and Characterization of TIP Antibodies

Synthetic peptides were prepared and conjugated with KLH by the MSU Macromolecular Structural Facility. The peptides were then injected in rat and chicken for antibody production by Cocalico Biologicals Company. The specificity of the antibodies was examined by western blot analysis of seed or leaf protein with 1:500 dilutions of each antibody in the presence of each synthetic peptide.

Obtaining Vacuoles from Arabidopsis Leaves

Plants that were homozygous for the *35S::GFP:Δ-TIP* transgene or wild type plants were grown on soil under long day conditions in a temperature controlled growth chamber. Protoplasts were isolated from leaves of 4-6 week old *Arabidopsis* plants using a protocol adapted from Damm & Willmitzer(1988) with modifications. Briefly, the

leaves were sliced into strips and digested for four hours at room temperature in the dark in a solution containing 1% cellulose R10, 0.5% Macerozyme R10, 30mM $CaCl_2$, 0.1% BSA, and 5mM 2-Mercaptoethanol. The resulting protoplasts were filtered through an 80μm sieve, then washed twice in 0.4M Mannitol and 10mM MES pH5.7 and centrifuged 20 minutes at 50xg in a swinging bucket rotor. Then the protoplasts were lysed and the vacuoles were purified according to a published protocol (Ahmed et al., 2000). The purity and concentration of vacuoles were determined by light microscopy with neutral red staining or fluorescent microscopy to check for GFP fluorescence.

Flow Cytometry

Fractions that contained a high concentration of vacuoles without debris were submitted for flow cytometry through the FACS Vantage Flow Cytometer (Becton Dickinson) at the MSU Core Flow Cytometry Facility. The 488 nm argon laser was used to excite GFP and the detector scanned for 530nm light. The sheath fluid was changed from PBS to a mannitol solution (0.5M Mannitol, 10mM Hepes pH 7.5, 1mM EDTA, 150mM NaCl) to meet the high osmolarity needs of the vacuoles.

Results

It has been suggested that distinct vacuoles within a cell can be distinguished from one another by the presence of specific Tonoplast Intrinsic Proteins (TIPs) on the tonoplast (Jauh et al., 1999). In order to be sure that TIPs decorate distinct vacuoles in *Arabidopsis*, I had to be able to specifically detect each TIP. In *Arabidopsis*, α-TIP, γ-TIP, and Δ-TIP are very similar to one another at the amino acid level; however, the C-termini are sufficiently divergent to be able to generate specific antibodies against each

TIP (Jauh et al., 1999). Peptides corresponding to the C-terminal amino acid residues of α-TIP (PPTHHAHGVHQPLAPEDY), γ-TIP (HEQLPTTDY), and Δ-TIP (HVPLASADF) were synthesized and coupled to Keyhole Limpet Hemocyanin (KLH) by the MSU Macromolecular Structural Facility. The peptides coupled to KLH were solubilized and injected into chicken and rat to raise antisera against the peptide. Chickens and rats were chosen so that double-immnocytochemistry could be done to ensure that multiple types of vacuoles in the same cell are distinguished by the TIPs present on the tonoplast.

The antibodies against α-TIP and γ-TIP from rat and chicken specifically recognized a band of the expected size (28kDa) in the seeds and leaves, respectively, and the antibody-antigen interaction could be competed away only in the presence of the peptide used to make the specific antibody (Figure C.1). The antisera raised against Δ-TIP from both rat and chicken do not specifically recognize Δ-TIP. These antibodies will allow us to determine whether distinct vacuoles in *Arabidopsis* can be distinguished from one another by the TIPs present on their tonoplast. They were (and are currently) also used in numerous other projects in Dr. Raikhel's laboratory.

Vacuoles Isolated From 35S::GFP:Δ-TIP Leaves have GFP Fluorescence in the Tonoplast

If different types of vacuoles can be distinguished by TIPs, then the TIPs will be used as markers to isolate each type of vacuole by FACS and examine the protein contents. The first question to address was whether isolated plant cell vacuoles could be visualized by fluorescence if a GFP-Tagged protein was localized to the tonoplast.

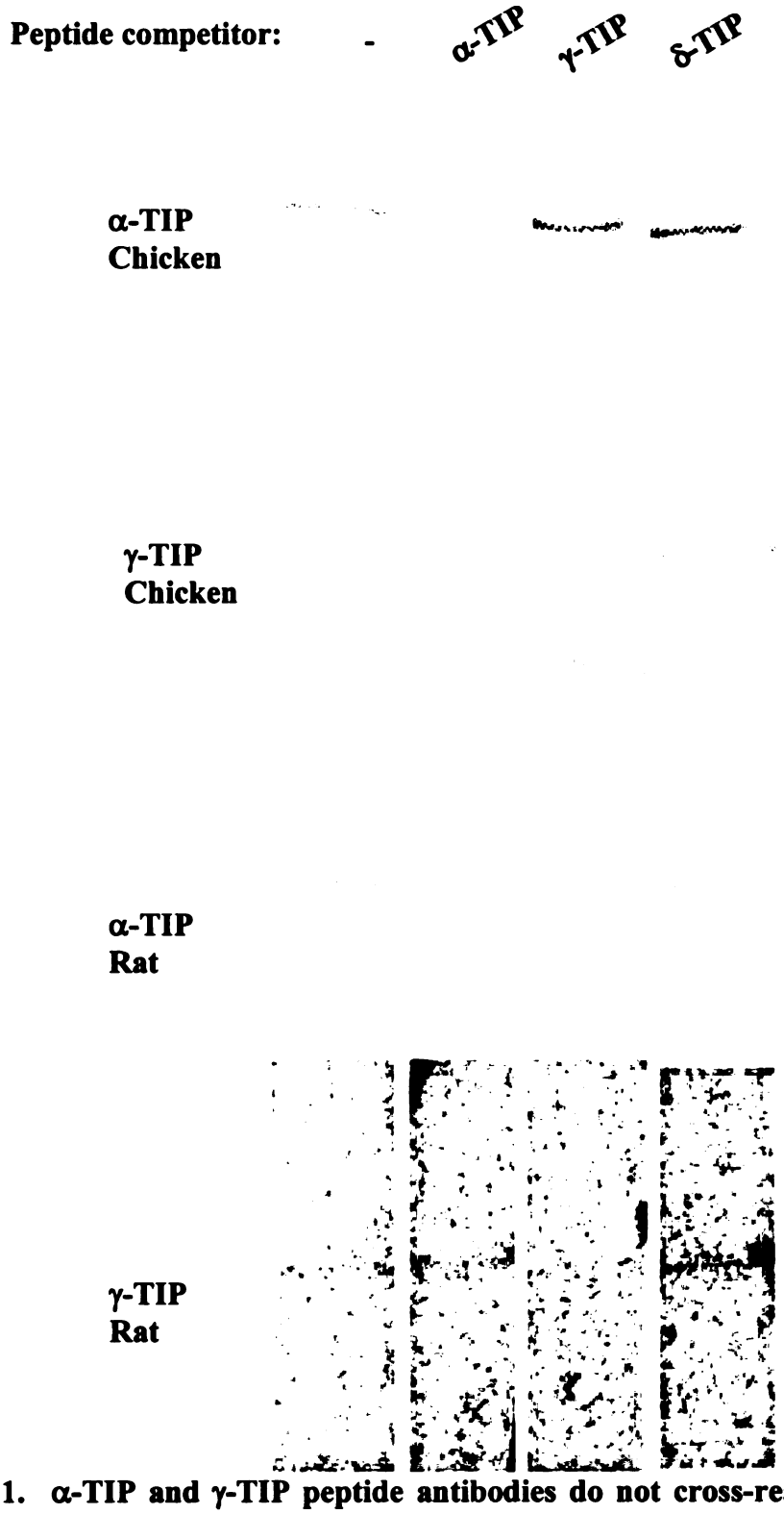

Figure C.1. α-TIP and γ-TIP peptide antibodies do not cross-react. *Arabidopsis* seed (α-TIP) or leaf (γ-TIP) protein extract was separated by SDS-PAGE and transferred to membrane. Each blot was incubated with a 1:500 dilution of the respective antibody that had been preincubated with the peptide competitor (1.2 μg/ml) for 1 hour. Then, each blot was rinsed well, incubated in secondary antibody, and visualized.

35S::GFP: Δ-*TIP* transgenic *Arabidopsis* seeds were kindly provided by Chris Somerville's laboratory to address this issue. The transgene in these plants is expressed in all tissues of the plant and the protein localizes to the tonoplast (Figure B.1) (Cutler et al., 2000). Therefore, vacuoles isolated from these plants should be recognized by GFP fluorescence in the tonoplast. To confirm this, we isolated vacuoles from leaves of the transgenic plants and looked at them by microscopy with Neutral Red staining and by fluorescence to observe GFP (Figure B.1). The isolated vacuoles stained red with Neutral Red and had GFP fluorescence, indicating that isolated vacuoles can be recognized by the presence of a GFP-linked marker protein in the tonoplast.

*Flow Cytometry of Vacuoles From Wildtype and 35S::GFP:*Δ*-TIP Plants*

I confirmed that GFP fluorescence was visible by microscopy in vacuoles isolated from 35S::GFP:Δ-TIP leaves. The next step was to determine whether a Fluorescence Assisted Cell Sorter (FACS) could also detect the fluorescence from isolated vacuoles. To accomplish this, I isolated vacuoles from wild type and *35S::GFP:*Δ*-TIP* transgenic leaves and compared them by flow cytometry in the FACS machine. Flow cytometry will scan the sample with the FACS detector but will not sort the sample into different populations. The results demonstrated that the FACS detector was able to distinguish vacuoles that contained GFP in their tonoplast from vacuoles that did not have GFP fluorescence (Figure C.2).

The next goal was to determine whether plant cell vacuoles can withstand the cell sorting procedure.

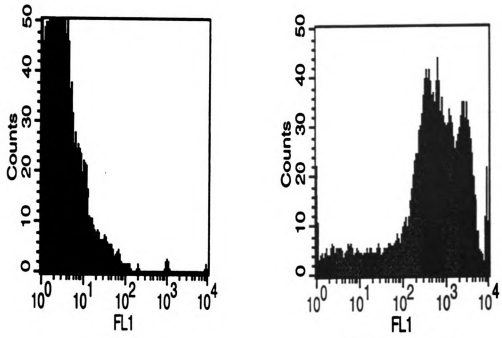

Figure C.2. Vacuoles that contain GFP in the tonoplast are recognized by flow cytometry. Vacuoles were isolated from leaves of wildtype and *35S::GFP::δ-TIP* transgenic plants and examined separately by flow cytometry. FL1 refers to the intensity of flourescence detected at 530 nm and counts refers to the number of times in which a specific intensity of fluorescence was detected.

The FACS was setup to separate fluorescent material away from the remaining fluid stream and was infiltrated with a mannitol sheath fluid to surround the vacuoles during the cell sorting procedure. The isolated vacuoles were placed into the FACS for sorting. For unknown reasons, the streams of fluid were periodically and randomly shifting to the left of the original stream and no healthy vacuoles were present in the separated fraction. When the mannitol solution was replaced with PBS, the stream-shift problem stopped, indicating that the problem was due to the mannitol solution. However, the isolated vacuoles were not stable in PBS due to its low osmolarity. These results suggested that vacuolar sorting through FACS might not be feasible with the current technology available.

Proteomics of Isolated Vacuoles

Since flow cytometry was not a feasible option, we decided to do a more global proteomic study of vacuoles isolated from leaf protoplasts. Protoplasts were isolated from healthy rosette leaves. Vacuoles were isolated from the protoplasts and the protein was extracted and analyzed by Syngenta (Table C.1). The purity of the vacuoles was determined visually by staining the sample with neutral red dye and examining the sample by microscopy.

Table C.1. Proteins Identified in Isolated Vacuoles

Arabidopsis Entry Number	Description and At Number	Size of Protein (Da)	Peptide (asterisk means that the Methionine is oxidized)
31456.t00004	At2g39460 60S ribosomal protein L23A	17441	1. K.KVNTLIRPDGTK.K 2. K.VNTLIRPDGTK.K
31701.T00008	At2g18960 Plasma membrane proton ATPase	104224	1. K.ESPGGPWEFVGLLPLFDPPR.H 2. R.GASDIVLTEPGLSVIISAVLTSR.A
40426.t00014	At2g35110 Unknown	148682	1. P.RVLESKKM*AK.S
40776.T00010	At2g47800 Glutathione-conjugate	169080	1. R.FGIIPQEPVLFEGTVR.S
48849.t00003	At2g18020 60S ribosomal protein L2	27859	1. K.GVVTEIIHDPGR.G
48852.t00004	At2g01250 60S ribosomal protein L7	28171	1. R.RVEPYVTYGFPNLK.S
50716.t00090	At1g23730 Putative carbonic anhydrase	28829	1. K.EAVNVSLGNLLSYPFVR.E
50729.t00024	At1g52040 Myrosinase binding protein	50167	1. K.AGDLVHQIGVHIVPIFTNY.R
50730.t00031	At1g61580 Ribosomal protein	44546	1. R.HGSLGFLPR.K
50968.t00011	Hypothetical protein	20216	1. K.KFETLSYLPDLTDSELAK.E
50978.t00048	At1g10150 Unknown protein	45518	1. K.DFLNSDSDEIPNSLKQIAKI.T
50980.t00030	At1g43170 Hypothetical protein	44559	1. K.GIQAQLEK.M
51028.t00050	At1g78900 Hypothetical protein similar to vacuolar ATP synthase catalytic subunit	68812	1. R.EDDLNEIVOLVGK.D 2. K.LTTFEDDEKESEYGYVER.K 3. R.NIIHFYNLANOAVER.A 4. K.YSTALESFYEKFDPDFINIR.T
51047.t00025	At1g65320 Unknown	46555	1. K.IIGEISASK.L
51096.t00045	At1g04270 Putative 40S ribosomal protein S15	17129	1. K.GVDLDALLDM*STDDLVK.L 2. R.NMIIVPEMIGSIIGVYNGK.T

51240.t00009	At1g23290 60S ribosomal protein 127a	16292	1. K.DNVPLIDVTQHGFFK.V
51416.t00012	At1g75940 Beta-glucosidase	61675	1. K.HWITFNEPWVFSR.A
51416.t00018	At1g76030 Vacuolar ATP synthase subunit B	54108	1. K.AVVGEEALSSEDLLYLEFLDKFER.K 2. M.KAVVGEEALSSEDLLYLEFLDKFER.K 3. R.NIFQSLDLAWTLLR.I 4. R.RGQVLEVDGEK.A 5. KYQEIVNIR.L
51595.t03791	At2g41560 Putative calcium-ATPase	112749	1. R.ITSISDIIEGFASEALR.T
51595.t03819	At2g41840 40s ribosomal protein S2	30879	1. K.EYQIIDM*LIGPTLKDEVMK.I 2. K.EYQIIDMLIGPTLKDEVM*K.I 3. K.EYQIIDMLIGPTLKDEVMK.I 4. K.KVLQFAGIDDVFTSSR.G 5. K.TYGFLTPEFWK.E 6. K.VLQFAGIDDVTSSR.G
51595.t04387	At2g47610 60S ribosomal protein L7A	29130	1. R.LKVPPALNQFTK.T 2. K.NEDKLEFSK.I 3. K.YGLNHVTYLIEQNK.A
51904.t00008	At1g12840 Vacuolar ATP synthase subunit C	41942	1. R.VGTLDSLLALGDDLLK.S
60008.t00025	At1g74060 Putative 60s ribosomal protein L6	26009	1. R.ASITPGTVLIILAGR.F 2. R.RVNQAYVIGTSTK.V
60047.t00013	At1g72610 Germin-like protein	21559	1. Q.TLKPGQVMVFPQGLLHFQ.I
60094.t00005	At1g52400 Beta-glucosidase	60459	1. F.HWDTPQDLEDEYGGFLSGR.I 2. K.IGIAHSPAWFEPQDLEHVGGSIER.V 3. R.SGYEAYQVSHNLLLSHAYAVDAFR.N 4. K.SPSWTTDSLVDWDSK.S 5. T.VFHWDTPQDLEDEYGGFLSGR.I 6. K.VGVQFYHDLIDELLK.N 7. R.VLDFILGWHLAPTTYGDYPQSM*.D 8. R.VLDFILGWHLAPTTYGDYPQSMK.D 9. L.VTVFHWDTPQDLEDEYGGFLSGR.I 10. K.WYSEFLKPQFPTSK.L
60185.t00009	At3g03780	84584	1. K.LNLPILPTTTIGSFPQTVELR.R

	Putative methionine synthase		
60208.t00029	At3g01500 Carbonic anhydrase, chloroplast precursor	36144	1. R.EAVNVSLANLLTYPFVR.E 2. K.EKYETNPALYGELAK.G 3. I.MSFPLDGNNSTDFIEDWVK.I 4. K.YGGVGAAIEYAVLHLK.V
60223.t00005	At3g04920 Putative ribosomal protein s19 or s24	15372	1. K.SSGFGLIYDTVESAK.K
60223.t00014	At3g04840 Putative 40s ribosomal protein s3a (S phase specific)	29851	1. K.ATQGIYPLQNVFIR.K
60244.t00006	At3g05590 Putative 60s ribosomal protein L18	20926	1. K.IAVLVGTITDDLR.V
60248.t00015	At3g07110 Putative 60S ribosomal protein L13A	23466	1. K.DLLNGQNIVVVR.C
60279.t00007	At3g04230 Putative 40S ribosomal protein S16	16586	1. K.IFEPVLLLGK.H
60480.t00017	At3g16240 Delta tonoplast integral protein (delta-TIP)	25027	1. M.AGVAFGSFDDSFSLASLR.A 2. K.GSLGTIAPLAIGLIVGANILAAGPFSGGSM*NPAR.S 3. K.GSLGTIAPLAIGLIVGANILAAGPFSGGSMNPAR.S
60518.t00009	At3g26520 Gamma tonoplast intrinsic protein (Gamma-TIP)	25849	1. R.NIAIGGVQEEVYHPNALR.A
60555.t00011	At3g28710 Adenosine triphosphatase	40792	1. R.YGHMIDNVVLIVTGTLHER.D
60557.t00008	At3g14210 Myrosinase-associated protein	44060	1. K.ANPNADASAQQAFVTNVINR.L 2. K.AQEEMAHLLYGADPDVVQPM.T 3. K.DLPQTYWPYGK.S 4. R.ELIVYPTGETMR.E 5. K.FSDGHIVPDFIADFISIPNGVLPPVLKPGVDIS.R 6. K.FSDGHIVPDFIADFISIPNGVLPPVLKPGVDISR.G 7. K.IGPMLNEFAK.I 8. K.ISTSPYGFQFTVFDFYNAVLR.R 9. LPNGVLPPVLKPGVDISR.G
60708.t00003	At3g28220 Unknown protein	42887	1. K.WTLPNFSSLEK.Q
61351.t00012	At3g31540	78613	1. K.SVEALADEM*GVTDK.S

	Hypothetical protein		
67109.t00023	At4g12160 Putative ribosomal protein	12127	1. K.LDYVLALTVENFLER.R
67182.t00001	At4g30800 Ribosomal protein S11	17931	1. K.NIGLGFK.T
67223.t00004	At4g39870 Putative protein	40961	1. K.SFRSKAVHFVTDLTAG.L
67224.t00001	At3g42050 Vacuolar H(+)-ATPase subunit-like protein	50284	1. R.AQLLDEDGPAYVHLFVSILR.D 2. R.DIFKEETVEYVLALIYEM*LSANPTR.A
67247.t00008	At3g45140 Lipoxygenase AtLOX2	99037	1. K.LDPAVYGDPTSLITWEIVER.E
67250.t00009	Putative protein	49859	1. -.MDFRKNQAGEKNVSSK.G
67265.t00003	At3g48930 Cytosolic ribosomal protein S11	17957	1. R.EAIDGAYVDKK.C
67268.t00026	At3g49910 60S ribosomal protein	16945	1. R.KDDEVQIVR.G
67286.t00012	At3g53420 Plasma membrane intrinsic protein 2a	30474	1. R.DYQDPPPAPFIDGAELK.K
67307.t00008	At3g58730 v-ATPase subunit D (vATPD)	29059	1. K.HVVLENVK.E
67577.t00009	At5g02780 Putative protein	28053	1. Q.RVWITRNLKGLQDEIKLVP.I
67583.t00017	At5g04570 Putative protein	62088	1. S.PPDKAKDYLLSIRGLG.L
67611.t00011	At5g10840 Putative protein	73286	1. R.LYKMFK.G
67620.t00010	At5g13650 GTP-binding protein type A (tyrosine phosphorylated protein A)	67534	1. K.AGLSPDDLAEDLGPLFEAIIR.C
67628.t00009	At5g15200 40S ribosomal protein-like	23036	1. R.DLLTLDEK.S 2. R.YGLLDESQNKLDYVLALTVENFLER.R
67905.t00007	At5g62250 Cytokinesis regulating protein-like	63869	1. S.HLILKRAEK.A
67913.t00005	At5g63600 1-aminocyclopropane-1carboxylic acid oxidase-like protein	36964	1. L.VIGAAAHSDMGAIALLIPNEVPGLQAFK.D

67951.t00012	At5g25980 Myrosinase TGG2	61353	1. R.GINEDGINYYSGLIDGLIAR.N 2. F.HWDLPQSLQDEYEGFLDR.T 3. K.YGDPLIYVTENGFSTSGGPIPFTEAFHDYNR.I
67991.t00004	At5g14740 Carbonic anhydrase 2	36615	1. K.YAGVGAAIEYAVLHLK.V
68070.t00281	Ribosomal protein	24239	1. R.VVNSYWLNEDSTYK.Y 2. K.YYEIILVDPAHNAVR.N
68082.t00018	At5g20290 Putative protein	24994	1. R.VLDVVYNASNNELVR.T
68096.t00004	At5g24770 Vegetative storage protein VSP2	29842	1. K.DYVEDYLITSK.Q 2. K.HLILKPNGSNLR.Q 3. W.IFDLDDTLLSSIPYYAK.Y 4. NIIGDQWADLVEDTPGR.V 5. K.KGYNIVGNIGDQWADLVEDTPGR.V 6. K.KGYNIVGNIGDQWADLVEDTPGRV.F 7. K.KGYNIVGNIGDQWADLVEDTPGRVF.K 8. Y.NIVGNIGDQWADLVEDTPGR.V 9. Y.QNIIELGIEPIILSDR.W 10. A.STPGLPEALHLYQNIIELGIEPIILSDR.W 11. K.TDPGAYWLWLGTGASTPGLPEALHLY.Q 12. N.VWIFDLDDTLLSSIPYYAK.Y 13. Y.WLWLGTGASTPGLPEALHLY.Q 14. Y.WLWLGTGASTPGLPEALHLYQNIIELGIEPIILSDR.W
68096.t00005	At5g24780 Vegetative storage protein VSP1	30262	1. K.AYVEDYLITSK.Q 2. Y.ENLLELGIEPIIISDR.W 3. K.HLILKPNGSK.L 4. K.LSEVTVENLK.A 5. E.SGESTPGLPETLHLYENLLELGIEPIIISDR.W 6. Y.WSWLESGESTPGLPETLHLY.E 7. Y.WSWLESGESTPGLPETLHLYENLLELGIEPIIISDR.W

Conclusion

Currently, FACS is not a feasible option for isolating and characterizing distinct types of plant cell vacuoles. Members of the laboratory are in collaboration with groups to identify the protein contents of general plant cell vacuoles by isolating vacuoles using the methods described above and submitting the vacuoles to multiple types of protein analysis techniques. Specifically, the goal is to identify proteins that differ between wildtype plants and mutant plants such as the *vpey* mutant. This strategy will allow us to identify potential substrates or cargo of known vacuolar transport machinery.

One possibility to separate different vacuoles that we are investigating is an optical trapping approach. Vacuoles isolated from plants with tonoplast fluorescent markers such as GFP would be isolated from plants and placed on a microscopic grid. The vacuoles will be monitored as they pass through the grid. When they reach a fork in the grid, fluorescent vacuoles will be sorted away from the non-fluorescent vacuoles by an optical trapping system. When a laser light of a specific intensity is focused on the vacuole, the light can actually direct its movement or stop its movement, effectively trapping the vacuole. With this method, specific types of vacuoles can be isolated from a general population of vacuoles and analyzed by proteomic techniques.

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Appendix D

Immunolocalization of PIN1 in Roots and Hypocotyls of *Arabidopsis* Seedlings

Introduction

Protein trafficking through the endomembrane system has been implicated in a variety of plant responses to the environment (Surpin and Raikhel, 2004). Vesicle trafficking through the endomembrane system requires the interaction of vesicle SNAREs (v-SNARE) and target membrane SNAREs (t-SNARE). One family of v-SNAREs that our lab has been studying is the AtVTI (Vps Ten-Interacting Factor) family composed of AtVTI11, AtVTI12, and AtVTI13. While *AtVTI11* and *AtVTI12* transcripts were found in a variety of tissues, *AtVTI13* is not expressed at detectable levels. The possibility that AtVTI11 and AtVTI12 are not redundant arose when our lab demonstrated that AVTI11 and AtVTI12 complemented different aspects of the Δ*vti* vacuolar trafficking phenotype in *S. cerevisiae* (Zheng et al., 1999). AtVTI11 colocalizes with an AtVSR protein at the TGN and PVC in *Arabidopsis* while AtVTI12 did not, further suggesting that AtVTI11 and AtVTI12 perform different functions (Zheng et al., 1999).

A mutant of AtVTI11 was independently identified in a mutant screen for components of shoot gravitropism (Kato et al., 2002). The stem of the *zig/sgr4/vti11* mutant grows in a "zig-zag" fashion, and it does not respond to a change in the gravity vector (Kato et al., 2002). However, a *vti12* mutant did not have any gravitropic phenotype (Zheng et al., 1999). In chapter three, I presented data indicating that plants that did not accumulate detectable levels of AtVSR protein had gravitropic defects (Chapter 3). Thus, the colocalization of AtVTI11 and AtVSR with the agravitropic phenotypes observed for mutants of *AtVTI11* as well as for the *AtVSR* family led us to hypothesize that AtVTI11 and a member(s) of the AtVSR family function together in a pathway that mediates gravitropism. To address this issue, I determined whether

AtVTI11 participated in the recycling of PIN1, a gravitropism pathway that has been previously characterized (Steinmann et al., 1999; Geldner et al., 2001; Geldner et al., 2003).

PIN1 is an auxin efflux carrier that localizes to the basal plasma membrane (Galweiler et al., 1998). It recycles to an endomembrane compartment and this recycling is inhibited by Brefeldin A, an inhibitor of endomembrane trafficking (Geldner et al., 2001). Therefore, PIN1 is dependent upon the endomembrane system for its localization at the basal plasma membrane and its recycling to an endosomal compartment. When PIN1 is absent from the basal plasma membrane, the plant exhibits auxin-related phenotypes, including defects in gravitropism (Geldner et al., 2001; Geldner et al., 2003). With this in mind, we speculated that AtVTI11 may play a role in the localization and recycling of PIN1. To test this hypothesis, I used confocal microscopy immunolocalization to determine whether the trafficking of PIN1 was blocked in *zig/vti11* roots and/or hypocotyls. Our lab published the results of many approaches taken to address the shoot gravitropic defect in *vti11* and determine the function of AtVTI12 (Surpin et al., 2003). My contribution to this project was the demonstration that AtVTI11 does not participate in the recycling of PIN1 between the basal plasma membrane and an endosomal compartment (Surpin et al., 2003). Therefore, AtVTI11 contributes to the negative gravitropism of stems via a pathway that is distinct from the PIN1-related pathway.

Materials and Methods

Immunolocalization of PIN1 in wildtype, *zig/vti11*, and *vti12* three-day old roots was performed essentially as described in Geldner et al. (2001). To immunolocalize PIN1 in three-day old hypocotyls, I followed the protocol described in Geldner et al. (2001) with the following modifications: *i)* 1% sucrose was added to the fixation buffer, *ii)* after adhering the hypocotyls to the slides, they were digested in 0.5% pectinase (Sigma) and 20% Triton X-100 (Sigma) at 37° C for 90 minutes. The samples were visualized with a Leica SP2 Confocal Microscope.

Results

In wildtype plants grown under normal conditions, the subcellular localization of PIN1 is at the basal plasma membrane in both the roots and the shoots of *Arabidopsis* (Geldner et al., 2001). Neither *zig/vti11* nor *vti12* mutants have root gravitropic defects (Zheng et al., 1999; Kato et al., 2002). Therefore, PIN1 should be localized to the basal plasma membrane of root cells in wildtype, *zig/vti11* mutants, and in *vti12* mutants. As expected, PIN1 localized to the basal plasma membrane of wildtype, *zig/vti11*, and *vti12* roots (Figure D.1, A-C).

The aerial tissues of *zig/vti11* mutants have gravitropic defects (Kato et al., 2002). However, the *vti12* mutant does not have any gravitropic defects (Zheng et al., 1999; Surpin et al., 2003). If VTI11 participates in the recycling of PIN1 to the basal plasma membrane to mediate gravitropism in aerial tissues, then PIN1 should be mislocalized in the *zig/vti11* mutant hypocotyls. Likewise, the *vti12* mutant is not agravitropic, and thus,

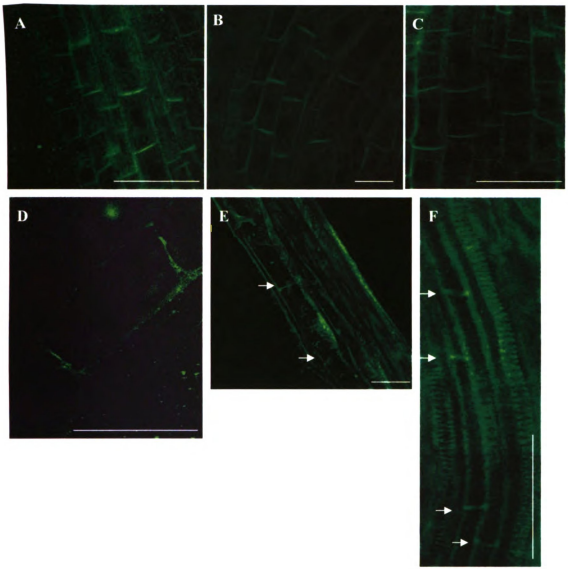

Figure D.1. PIN1 localized to the basal plasma membrane in *zig/vti11* and *vti12*. Antibodies against PIN1 were used to determine its subcellular localization in three-day old seedling roots (A-C) and hypocotyls (D-F) of wildtype (A, D), *zig/vti11* (B, E), and *vti12* (C, F) plants. Arrows indicate basal localization of the PIN1 signal.

PIN1 should correctly localize to the basal plasma membrane in *vti12* hypocotyls. To test this hypothesis, I determined the localization of PIN1 in wildtype, *zig/vti11*, and *vti12* hypocotyls by confocal microscopy immunolocalization. Surprisingly, I found that PIN1 localized to the basal plasma membrane in all seedling hypocotyls (Figure D.1, D-F). Therefore, AtVTI11 is not the v-SNARE present on the vesicles that recycle PIN1 between the basal plasma membrane and an endosomal compartment.

Discussion

The goal of this experiment was to determine whether the gravitropic defect observed in *zig/vti11* plants was due to the mislocalization of PIN1 protein. By my observations, this was not the case. However, other experiments discussed in the paper indicate that there is an auxin transport defect in the *zig/vti11* plants (Surpin et al., 2003). Therefore, AtVTI11 is required for auxin transport, but that requirement is not linked to the proper localization of PIN1 (Surpin et al., 2003). PIN3 is involved in auxin transport in addition to PIN1 (Geldner et al., 2001; Friml et al., 2002). Therefore, it is possible that PIN3 is mislocalized in *zig/vti11* mutants. Another possibility arises from the observation that *zig/vti11* plants have defects in tissue identity and organization (Surpin et al., 2003). Gravitropism is mediated from the endodermal tissues (Surpin and Raikhel, 2004). Therefore, it is possible that the misorganization of cell types in *zig/vti11* plants causes the gravitropic defect (Surpin et al., 2003). These alternative explanations have not been tested yet. Furthermore, the link between the VSRs and gravitropism has not been established yet either. Further inquiry into the connection between VTI11, AtVSRs,

and shoot gravitropism is clearly necessary to obtain a complete understanding of protein trafficking through the endomembrane system and gravitropism.

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