This is to certify that the

dissertation entitled

Identification of Components Involved in Plant Vacuolar Protein Targeting

presented by

James Edward Dombrowski

has been accepted towards fulfillment
of the requirements for

PhD. degree in Genetics

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Date July 31, 1995

IDENTIFICATION OF COMPONENTS INVOLVED IN PLANT VACUOLAR PROTEIN TARGETING

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Genetics Program

1995

ABSTRACT

IDENTIFICATION OF COMPONENTS INVOLVED IN PLANT VACUOLAR PROTEIN TARGETING

By

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The main focus of current research is the investigation of the molecular mechanisms of protein transport to the plant cell vacuole. We have previously demonstrated that the carboxyl-terminal propeptide of barley lectin is both necessary and sufficient for protein sorting to the plant vacuole. Specific mutations were constructed to determine which amino acid residues or secondary structural determinants of the carboxyl-terminal propeptide affect proper protein sorting. The experimental results obtained from the detailed mutational analysis of barley lectin's carboxyl-terminal propeptide, revealed that no consensus sequence or common structural determinants are required for proper sorting of barley lectin to the vacuole. However, the analysis did show the importance of hydrophobic residues in vacuolar targeting. In addition, a minimal length of three exposed amino acid residues are necessary for efficient sorting. Sorting was disrupted by the addition of two glycine residues at the carboxyl-terminal end of the targeting signal or by the translocation of the

glycan to the carboxy terminus of the propeptide. These results suggest that some components of the sorting apparatus interact with the carboxy terminus of the propeptide.

One approach to identify components of the sorting apparatus in plants is to look for homologous proteins involved in protein vacuolar transport which have been isolated from yeast. A cDNA encoding for a 68 kDa GTP binding protein was isolated from *Arabidopsis thaliana* (aG68) and characterized. This clone is a member of a larger gene family that codes for a class of several GTP binding proteins this includes the mammalian dynamin, yeast Vps1p and the vertebrate Mx proteins. In yeast the *VPS1* gene has been shown to be involved in retention of proteins in the Golgi and protein transport to the yeast vacuole. The investigation of *aG68*'s function in *Arabidopsis* is currently in progress.

The development of a preliminary genetic screen for the identification of vacuolar protein sorting mutants in *Arabidopsis*, has identified three putative mutants. This initial mutant screen demonstrated the feasibility of using *Arabidopsis* as a model system. The information gathered from this study has led to the development of a second generation mutant screen utilizing a double transgene approach.

DEDICATION

To my parents
Irene and Edward Dombrowski

ACKNOWLEDGEMENTS

I would like to thank my grade school teacher, Ms. Gilbert, for believing in me. Natasha Raikhel for her friendship, and for helping me discover what it means to be a research scientist. I would also like to thank the following: my committee members, Dr. Ken Keegstra, Dr. Michael Thomashow and Dr. William Smith for their many helpful suggestions over the years. The office staff, Karen Bird, Jan Johnson, Jackie Malkin, Karen Cline and Alice Albin for their warmth and professionalism. My good friends Mark Shieh, Susan Fujimoto and Elizabeth Rosen. Marlene Cameron, Kurt Stepnitz, Glenn Hicks, Karen Bird, Diane Bassham, Silvia Rossbach, Sridhar Venkataraman and Olga Borkhsenious for their valuable assistance in the preparation of this thesis. All my Lab comrades of the ZONE, past and present. All graduate students everywhere, who live by the creed; "Life without liberty, in the pursuit of your professor's happiness." The LORD up above, who helped me make it through each day. My parents for instilling in me their values, and for their sacrifices that gave me a chance in life. My cats, Bunky, MooMoo, and Wompee, who were always there to comfort me. And finally to my wife, Maria, whose patience and love has made each day worth living.

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CHAPTER 1

PROTEIN TARGETING TO THE PLANT VACUOLE

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INTRODUCTION

The plant vacuole is a dynamic multifunctional organelle that is essential for the regulation and maintenance of plant cell growth and development. The vacuolar composition and morphology, and the number of vacuoles per cell varies among plant tissues and developmental stages. Vacuoles in many cells act as intermediate storage compartments for ions, sugars, amino acids, secondary metabolites and proteins (Marty et al., 1980; Boller and Wiemkem, 1986; Wink 1993). Defining the content of vacuoles gave early clues to their functions (Boller and Kende, 1979). These early studies of vacuoles led to the hypothesis that they were ultimately derived from the endoplasmic reticulum (ER); however, more recent work indicates that vacuoles originate from various parts of the endomembrane system, such as the ER and Golgi complex (Hubbard and Ivatt, 1981; Chrispeels, 1983; for reviews Marty et al., 1980; Boller and Wiemkem, 1986; Harris, 1986). In addition, protein and oil bodies derive from components of the secretory pathway (discussed in Bednarek and Raikhel, 1992).

A wide array of functions associated with the vacuole are performed by proteins. Some vacuolar proteins arrive at the tonoplast or enter the vacuole via novel routes (Dice, 1990; Tranbarger et al., 1991; Yoshihisa and Anraku, 1990; Monroe et al., 1991), while other proteins enter the vacuole by internalization of ER-derived protein bodies by a process analogous to autophagy (Leavanony et al.,1992; Galili et al.,1993; Galili et al., 1995; Robinson et al., 1995).

However, the majority of proteins are delivered to the vacuole by way of the secretory pathway. (reviews: Pfeffer and Rothman, 1987; Chrispeels, 1991; Bednarek and Raikhel, 1992; Vitale and Chrispeels, 1992; Nakamura and Matsuoka, 1993; Satiat-Jeunemaitre and Hawes, 1993; Rothman and Orci, 1992; Rothman, 1994).

Proteins are targeted to the secretory pathway by amino-terminal (N-terminal) hydrophobic signal peptides which mediate transmembrane translocation from the cytosol to the lumen of the ER (for reviews see Rapoport, 1992; Gilmore, 1993; Walter and Johnson, 1994). These signal peptides are usually 18-30 amino acids long, have no consensus sequence and can function interchangeably between animal, yeast, and plant systems (von Heijne, 1988, 1990; Jones and Robinson 1989). However, these sequences do possess common secondary structural features, a basic N-terminal region followed a hydrophobic region of amino acids (von Heijne, 1990). In general, signal peptides are cotranslationally removed, and the resulting polypeptide is processed in the ER and Golgi network (Blobel and Dobberstein 1975; Morrè, 1987; Vitale et al.,1993).

It was initially believed that after entering the lumen of the ER, proteins traveled to the Golgi apparatus by bulk flow (Wieland et al., 1987; Rothman, 1987; Pelham, 1989; Griffiths et al., 1995) which is mediated by vesicle budding and fusion events (Pryer et al., 1992; Rothman and Orci, 1992; Ferro-Novick and Jahn, 1994; Rothman, 1994). However, more recent findings suggest that protein exit from the ER is a regulated process. Before leaving the

ER proteins must first pass stringent quality-control mechanisms (Hurtley, 1989; Vitale et al., 1993; Li et al., 1993), then they are concentrated in the ER (Mizuno and Singer, 1993; Balch et al., 1994; Balch and Farquhar, 1995; Singer, 1995) before transport to the Golgi. In addition, there is evidence in yeast which suggests that proteins are packaged into transport vesicles differentially (Barlowe et al., 1994; Schimmöller et al, 1995). Proteins traversing the secretory pathway are believed to be sorted by selective retention or targeting information contained in their molecular structures (Blobel, 1980; Rothman, 1987; Pelham, 1989). Proteins lacking specific sorting determinants follow a default pathway and are secreted at the cell surface (Rothman, 1987; Wieland et al., 1987; Dorel et al., 1989; Denecke et al., 1990). After traversing the Golgi apparatus (Rambough and Clermont, 1990) vacuolar proteins are sorted at the *trans*-Golgi Network (TGN) and targeted to the vacuole (Griffiths and Simons, 1986; Morrè, 1987).

Vacuolar proteins are sorted at the trans-Golgi Network and transported by clathrin coated vesicles

Two lines of evidence suggest that many plant vacuolar proteins which traverse the secretory pathway are sorted at the *trans*-Golgi Network (TGN). First, precursors of vacuolar seed storage proteins were found in clathrin coated vesicles (CCVs) isolated from developing legume cotyledons (Harley and Beevers, 1989; Robinson et al., 1989; Hon et al., 1991). Electron microscopic

examination had shown that CCVs are associated with the TGN (Staehelin, 1990; Driouch et al., 1993a).

Secondly, relevant information was obtained by the use of inhibitors which affect golgi structure. Ultrastructural observations showed that when plant and animal cells were treated with the fungal toxin monensin, a monovalent cation ionophore, the formation of secretory vesicles at the TGN was inhibited, causing the cisternae to swell (Grimes et al., 1982; Tartakoff, 1983; Stinissen et al., 1985; Zhang et al., 1993; for a review see Mollenhauer et al., 1990). This effect may be due to disruption of the correct balance of cations within the affected cisternae and the proton gradient across the membrane. Monensin was found to inhibit transport of pea vicilin (Craig and Goodchild, 1984), concanavalin A (con A) (Bowles et al., 1986), phytohemagglutinin (PHA) (Chrispeels, 1983), probarley lectin (Wilkins et al., 1990) and prosporamin (Nakamura et al., 1993) to the vacuole. However the effect of monensin on these missorted proteins differed. Con A, pea vicilin and prosporamin were secreted from cells treated with monensin, whereas PHA and probarley lectin were retained intracellularly. Monensin also displays differential effects on the secretion of complex polysaccharides (discussed in Bednarek and Raikhel, 1992).

Another fungal toxin, brefeldin A (BFA), was shown to severely disrupt the organization of the mammalian Golgi stack (for a review see Klausner et al., 1992). Recently, Satiat-Jeunemaitre and Hawes summarized the effects of BFA in plants (Satiat-Jeunemaitre and Hawes, 1994). The current data indicates that

BFA will inhibit transport of vacuolar proteins and disrupt the architecture of the Golgi. However, it should be noted that the effects of BFA varied among plant species and cell types. One possible explanation for this variation may be to differential regulation of the activity of the Golgi complex within the different tissues and cell types. Recent ultrastructural analyses of plant Golgi indicate that there are differences in the morphology and the number of cisternal stacks within individual cells (Staehelin et al., 1988; Staehelin et al., 1990; Zhang and Staehelin, 1992; Driouch et al., 1993 a,b).

Sorting signals of vacuolar proteins.

Glycans (mammalian)

Extensive research has been performed over the last 10 years to define the sorting determinants of lysosomal and vacuolar proteins. The best-characterized targeting signal is the mannose-6-phosphate residue that specifies transport of hydrolytic enzymes to the mammalian lysosome. The targeting of these enzymes to the lysosome is mediated by the phosphorylated mannose residue of an N-linked glycan, which interacts with receptors in the TGN (reviews: Hasilik and Neufeld, 1980: von Figura and Hasilik, 1986; Kornfeld and Mellman, 1989; Dahms et al., 1989; Kornfeld, 1992; Fiedler and Simons, 1995). However, there also appears to be a mannose-6-phosphate-independent pathway for the delivery of proteins to the lysosome (Kornfeld and Mellman, 1989; Rijnbout et al., 1991; Glickman and Kornfeld, 1993; Garcia-del Portillo

and Finlay, 1995).

It was observed that the carbohydrate chains of the yeast vacuolar hydrolase carboxypeptidase Y (CPY) are phosphorylated (Hashimoto et al., 1981). CPY is a glycoprotein that is synthesized as an inactive preproenzyme (Hasilik and Tanner, 1978; Stevens et al., 1982), whose signal sequence is subsequently removed in the ER (Johnson et al., 1987). During its transport through the Golgi its core oligosaccharides are modified, and the mature protein is formed by a postsorting proteolytic activation via the vacuolar enzyme proteinase A (Hemmings et al., 1981; Ammerer et al., 1986; Woolford et al., 1986). Studies in which the attachment of glycans to CPY was blocked with the inhibitor tunicamycin (for a review see Elbein, 1987), showed that neither the phosphorylated sugar residues nor the presence of the glycans were required for proper delivery of the hydrolase to the yeast vacuole (Schwaiger et al., 1982; Stevens et al., 1982; Klionsky et al., 1988).

In contrast to mammalian and yeast systems, the phosphorylation of carbohydrate sidechains of plant glycoproteins has not been detected (Gaudreault and Beevers, 1984; Vitale and Chrispeels, 1984). However, it was still unknown if glycans had a role in the targeting of plant vacuolar proteins (Faye et al., 1989). Four vacuolar proteins with different glycan configurations were studied to investigate the functional role of the glycan in processing and intracellular transport. Barley lectin (BL) is a homodimeric vacuolar protein that specifically binds to the sugar *N*-acetylglucosamine (for a review see Raikhel and Lerner, 1991). BL is synthesized as a preproprotein with a high-mannose

glycosylated 15 amino acid carboxy-terminal propeptide (CTPP) that is removed before or concomitant with deposition of the mature protein into the vacuole. Phytohemagglutinin (PHA) is a tetrameric glycoprotein, with each subunit containing one high-mannose and one complex glycan. PHA accumulates in the protein storage vacuole of developing bean embryos (Bollini and Chrispeels, 1978; Chrispeels, 1983; Strum et al., 1988). Another vacuolar protein, patatin from potato tubers, contains two N-linked complex glycans in the mature protein (Sonnewald et al., 1989). Each of these proteins were transformed into tobacco and found to be correctly processed and targeted to the vacuole. One other protein studied was concanavalin A (Con A), a tetrameric lectin that is synthesized as a glycosylated precursor and undergoes a proteolytic circular permutation and loss of a small glycopeptide upon maturation in the vacuole (Bowles et al., 1986; Herman et al., 1985; Faye and Chrispeels, 1987). The inhibition of glycosylation by the use of the inhibitor tunicamycin or by the elimination of the glycosylation site by site-directed mutagenesis did not interfere with the proper targeting of BL (Wilkins et al., 1990), PHA (Bollini et al., 1985; Voelker et al., 1989) or patatin (Sonnewald et al., 1990) to the vacuole. However, the inhibition of glycosylation of Con A prevents its transport from the lumen of the ER (Faye and Chrispeels, 1987). In addition, it appears that the glycan of the CTPP of BL influences the rate of posttranslational processing of the propeptide (Wilkins et al., 1990). In conclusion, unlike mammalian systems, it appears that the targeting of proteins to the vacuoles of yeast and plants is independent of glycosylation.

Signals in yeast

In yeast, a number of soluble vacuolar proteins are synthesized as glycosylated higher molecular weight precursors (for review see Klionsky et al., 1990). The best characterized example is the protease carboxypeptidase Y (CPY), discussed above, which is synthesized with a 91 amino acid amino-terminal propeptide (NTPP) that is removed in a post-sorting event. Since the glycan was found not to be involved in sorting to the vacuole, research was directed towards determining if the NTPP of CPY contained sorting information. A deletional analysis of the NTPP showed that it was necessary for the correct sorting of CPY (Valls et al., 1987) and was sufficient to redirect the secreted protein, invertase, to the yeast vacuole (Johnson et al., 1987). The region of the CPY propeptide responsible for vacuolar targeting was determined by deletion analysis to reside in a 16 amino acid region. Further analysis of this region of the propeptide identified a tetrapeptide, QRPL, located near the amino-terminus of the propeptide, which functions as the vacuolar targeting signal. It was also found that the context in which the QRPL sequence is presented can affect the efficiency of targeting, implying that secondary structural determinants are also involved (Valls et al., 1987, 1990). Another yeast vacuolar targeting domain for the hydrolase proteinase A has been identified based on its ability to redirect invertase to the vacuole (Klionsky et al., 1988). This targeting determinant is also located in the propeptide; however, it contains no significant sequence similarity to the CPY sorting domain. It is

interesting to note that even when the entire propeptide was deleted, a small amount of proteinase A was correctly targeted to the vacuole, indicating that a secondary targeting signal may also be present in the mature portion of the protein. A third soluble yeast vacuolar protein, proteinase B, contains the sequence QNPL in its propeptide, however the propeptide is cleaved in the ER and has not been shown to act as a targeting signal (Moehle et al., 1989).

Recently the yeast CPY-specific sorting receptor was identified. This receptor is encoded by the *VPS10* gene (Marcusson et al., 1994). The *vps10* mutant selectively missorts and secretes CPY, whereas all other vacuolar proteins tested are correctly delivered to the vacuole. The *VPS10* gene encodes a type I transmembrane protein of 1577 amino acids. Chemical cross-linking studies have indicated that the Golgi-modified form of CPY interacts with VPS10p. The identification of this receptor combined with the fact that no consensus sequence or structural determinant has been demonstrated for vacuolar targeting in yeast, suggests that there are multiple receptors involved in the sorting process.

NTPP signals in plants

A number of vacuolar proteins have been identified which have NTPPs (Figure 1.1). A comparison of these sequences show that they all share a common motif, NPIRP\L. The presence of a conserved amino acid sequence suggests that this motif is a vacuolar sorting determinant (for reviews, see

FIGURE 1.1 Common Motif in Representative N-terminal Propeptides.

Codes: V, protein is vacuolar localized; pV, putative vacuolar protein; N, sequence necessary for proper sorting to vacuole; S, sequence sufficient to redirect a reporter protein to vacuole.

References: 1) Hattori et al., 1985; 2) Matsuoka and Nakamura, 1991; 3) Matsuoka et al., 1995; 4) Roger et al., 1985; 5) Holwerda et al., 1992; 6) Yamagishi et al., 1991; 7) Strukeij et al., 1990; 8) Watanabe et al., 1991.

Common Motif in Representative N-terminal Propeptides

PROTEIN	*SEQUENCE*	*CODES*	*REFERENCES*
Sweet potato sporamin A	..HSRFNPIRLPTTHEPA..	V,N,S	1,2,3
Barley aleurain	..SSSSFADSVNPIRPTDRAASTLE..	V,N,S	4,5
Potato 22 kDa protein	..FTSENPIVLPTTCHDDN..	pV	6
Potato cathepsin D inhibitor	..FTSQNLIDLPS..	pV	7
Rice oryzains	..ASSGFDDSNPIRSVTDHAASA..	pV	8

Bednarek and Raikhel, 1992; Chrispeels and Raikhel, 1992).

Sporamin is a vacuolar storage protein of the tuberous roots of sweet potato and is synthesized as a preproprotein, which contains a 16 amino acid propeptide that follows the N-terminal signal sequence (Hattori et al., 1985, 1987; Maeshima et al., 1985). Sporamin has been shown to be correctly processed and targeted to the vacuoles of transgenic tobacco and BY-2 suspension cell cultures (Matsuoka et al., 1990). Deletion of the NTPP results in the secretion of sporamin in transgenic tobacco cells (Matsuoka and Nakamura, 1991). In order to determine the region or specific amino acids in the NTPP that are responsible for proper targeting, glycine substitution and deletional analyses were performed (Nakamura et al., 1993). As a result of the site-directed mutagenesis, it was found that 2 residues, asparagine^{26} and isoleucine^{28} play an important role in the transport of sporamin to the vacuole. The substitution of glycine for asparagine and isoleucine caused the secretion of 40% and 90% of prosporamin, respectively. The asparagine and isoleucine residues appear to be conserved among all the currently identified NTPPs of vacuolar proteins (Figure 1.1).

The vacuolar thiol protease aleurain from barley is also synthesized as a higher molecular weight precursor that undergoes proteolytic processing to the mature form after a post-sorting event (Rogers et al., 1985). When expressed in tobacco, aleurain was shown to be correctly processed and targeted to the vacuole (Holwerda et al., 1990). Deletion and redirection analyses demonstrated

that the targeting information in aleurain resides in the NTPP (Figure 1.1) (Holwerda et al., 1992; Holwerda and Rogers, 1993). These studies identified two adjacent sequences, SSSSFADS and SNPIR, within the NTPP that were able to redirect the normally secreted endoprotease B (Koehler and Ho, 1990) to the vacuole. It was also shown that the presence of additional sequences contained in the propeptide had a strong effect on targeting efficiency. The combined sequences targeted more efficiently than the sum of the separate determinants. These results indicate that not only is the presence of the sorting determinant required for proper targeting, but also the context in which it is presented to the sorting machinery. This is similar to yeast CPY described above, where it was shown that the sequence surrounding the sorting determinant affected the efficiency of sorting.

CTPP signals in plants

Many vacuolar proteins have carboxyl-terminal propeptides (CTPPs) that share no common sequence identity, but contain short stretches of hydrophobic amino acids (Figure 1.2) (for reviews see Bednarek and Raikhel, 1992; Chrispeels and Raikhel, 1992).

Barley lectin is synthesized as a preproprotein with a high-mannose glycosylated 15 amino acid carboxy-terminal propeptide (CTPP) that is removed before or concomitant with deposition of the mature protein into the vacuole.

FIGURE 1.2 Representative Sequences of Carboxy-terminal Propeptides and Extensions of Vacuolar and Putative Vacuolar Proteins.

Codes: V, protein is vacuolar localized; pV, putative vacuolar protein; N, sequence necessary for proper sorting to vacuole; S, sequence sufficient to redirect a reporter protein to vacuole.

Propeptide: a region of amino acids that is proteolytically removed to form the mature protein.

Extension: a region of amino acids which has been identified by sequence comparison of cDNAs of intracellular and extracellular isoforms of a protein.

References: 1) Lerner and Raikhel, 1989; 2) Bednarek et al., 1990; 3) Bednarek and Raikhel, 1991; 4) Shinshi et al., 1990; 5) Neuhaus et al., 1991; 6) Shinshi et al., 1988; 7) Linthorst et al., 1990; 8) Melchers et al., 1993; 9) Melchers et al., 1993; 10) Wilkins and Raikhel, 1989; 11) Benatti et al., 1991; 12) Podivinsky et al., 1989; 13) Paul et al., 1995; 14) Legname et al., 1991; 15) Unger et al., 1994; 16) Johansson et al., 1992; 17) Payne et al., 1989; 19) Dixon et al., 1991.

Representative Sequences of Carboxy-terminal Propeptides and Extensions of Vacuolar and Putative Vacuolar Proteins

PROTEIN	SEQUENCE	CODES	REFERENCES
Propeptides			
Barley lectin	..VFAEAIAANSTLVAE	V,N,S	1,2,3
Tobacco chitinase	..GLLVDTM	V,N,S	4,5
Tobacco β-1,3-glucanase	..VSGGVWDSSVETNATASLVSEM	V,N	6,7,8
Tobacco AP24	..QAHPNFPLEMPGSDEVAK	V,N	9
Rice lectin	..DGMAAILANNQSVSFEGIIESVAELV	V	10
Soapwort saporin 6	..SSNEANSTVRHYGPLKPTLLIT	pV	11
Kiwifruit actinidin	..QNHPKPYSSLINPPAFSMSKDGPVGVDDGQRYSA	V	12,13
Extensions			
Carnation dianthin 30	..PKSSSIEANSTDDTADVL	pV	14
Carrot β-fructofuranosidase II	..HFADLVI	pV	15
Barley peroxidase BP1	..LLHDMVEVVDFVSSM	pV	16
Tobacco PRB1b	..GDLEEQHPFDSKLELPTDV	V	17,18
Arabidopsis RNS2	..LDGEAMVLKMPTEREAL	PV	19

The removal of the CTPP from BL is proposed to be carried out by an aspartic proteinase (Runeberg-Roos et al., 1994). The CTPP of BL is rich in hydrophobic residues, has 2 glutamic acid residues, and has the potential to form an amphipathic α-helix. The expression of a cDNA clone encoding this monocot protein in transgenic tobacco, a dicot plant, resulted in correct processing, maturation and accumulation of active BL in vacuoles (Wilkins et al., 1990). This correct sorting of BL suggests that similar mechanisms for vacuolar protein transport exist in both monocot and dicot plants. Pulse-chase analysis, electron microscopy (EM) immunolocalization and cell fractionation studies were used to determine if the CTPP contained the sorting determinant. These studies demonstrated that the CTPP is necessary for proper sorting of BL (Bednarek et al., 1990) and is sufficient to redirect a secreted protein, cucumber chitinase (Boller and Métraux, 1988) to the vacuole of transgenic tobacco plants (Bednarek and Raikhel, 1991). Therefore, the CTPP of BL contains vacuolar targeting information within its sequence or structure.

The experimental results obtained from a detailed mutational analysis of barley lectin's CTPP, revealed that no consensus sequence or common structural determinants are required for proper sorting of BL to the vacuole (Dombrowski et al., 1993). However, the analysis did show the importance of hydrophobic residues in vacuolar targeting. In addition, a minimal length of 3 exposed amino acid residues are necessary for efficient sorting. Sorting was disrupted by the addition of two glycine residues to the carboxyl-terminal end of the targeting signal or by translocation of the glycan to the carboxy-terminus

of the propeptide. These results suggest that the component of the sorting machinery which recognizes the CTPP interacts with the carboxy-terminal end of the propeptide.

Similar results were obtained from the analysis of the seven amino acid CTPP of *Nicotiana tabacum* chitinase A. This CTPP was also shown to be necessary for the transport of chitinase A to the vacuoles of *Nicotiana silvestris* and sufficient for to redirect the normally secreted cucumber chitinase to the plant vacuole (Neuhaus et al., 1991). A mutational analysis of the chitinase A CTPP, combined with its substitution by random sequences, illustrated that sequence changes in the CTPP allowed for a gradual transition from vacuolar retention of chitinase A to its secretion from the cell (Neuhaus et al., 1994). The most dramatic effect in the secretion of chitinase A by a single amino acid exchange was observed when the methionine of the CTPP (Figure 1.2) was replaced by a glycine. In addition, when extremely high levels of chitinase A were transiently produced in *Nicotiana plumbaginifolia* protoplasts, secretion of chitinase A was observed, suggesting saturation of the sorting system and the receptor.

Thus, the extensive mutational analysis of the fifteen amino acid CTPP of BL and the seven amino acid CTPP of chitinase A yielded comparable results. These two analyses confirmed that the recognition of highly variable sequences by the sorting apparatus was not a phenomena specific to BL, but a general characteristic. The highly polymorphic nature of CTPP targeting signals can also be seen by comparing the sequences of CTPPs of various vacuolar proteins and

C-terminal extensions of putative vacuolar proteins displayed in Figure 1.2. In conclusion, the component of the sorting apparatus that interacts with the CTPP binds to short sequences of amino acids, has low sequence specificity and interacts with the C-terminus of the propeptide.

Targeting information in mature portions of plant proteins

Some soluble plant vacuolar proteins are synthesized without cleavable propeptides, indicating that the sorting information is contained in a portion of the mature protein. The lectin PHA of common bean accumulates in protein storage vacuoles. Except for the removal of the signal sequence it does not undergo any additional proteolytic steps (Bollini et al., 1985). Initial studies to define a vacuolar targeting signal for PHA used yeast as a model system. When expressed in yeast, full length PHA was correctly sorted to the vacuole (Tague and Chrispeels, 1987). In order to identify the vacuolar sorting information contained in PHA, a series of gene fusions were constructed containing portions of the mature PHA protein fused to the secreted yeast protein invertase. This analysis identified a short region of amino acid residues (14-23) that contained the sorting information (Tague et al., 1990). Sequence analysis indicates that this domain contains the yeast-like targeting sequence LQRD. Mutations in the LQRD sequence caused increased levels of secretion in certain PHA-invertase hybrid proteins. However, similar alterations in the longer PHA-invertase fusions or the full length PHA did not yield dramatic effects on the level of secretion of

PHA in yeast (Tague et al., 1990). Thus, although this sequence is sufficient for targeting to the yeast vacuole, it is not necessary. However, when some of these same PHA-invertase constructs were expressed in *Arabidopsis thaliana* protoplasts, they failed to yield significant vacuolar localization (Chrispeels, 1991). Therefore, the sorting determinant contains enough information for proper sorting in yeast but appears to lack the necessary information for efficient targeting in plants. Further analysis of PHA in plants revealed an additional protein segment between amino acids 84-113 which acts as the targeting determinant in plants (von Schaewen and Chrispeels, 1993).

The vacuolar glycoprotein patatin, from potato tubers, is correctly targeted to the vacuole of transgenic tobacco (Sonnewald et al., 1990). Other than the removal of the signal sequence, patatin undergoes no additional proteolytic processing. When the N-terminal 146 amino acids of the protein, including the 23 amino acid signal sequence and 123 amino acids of the mature protein, were fused to the yeast invertase (von Schaewen et al., 1990), the chimeric protein was redirected to the vacuoles of transgenic tobacco (Sonnewald et al., 1991). This demonstrated that a portion of the mature protein is sufficient to redirect a secreted protein to the plant vacuole.

The hexameric 11S globulin legumin is a bean storage protein that accumulates in the cotyledon cell storage vacuole. This protein is also correctly delivered to the vacuoles in tobacco. After entering the ER, the 11S polypeptides form trimers that are transported by way of the Golgi apparatus to the vacuole (for review see Akazawa and Hara-Nishimura, 1985). In order to

study its targeting, chimeric fusion proteins consisting of the reporter protein chloramphenicol acetyl transferase (CAT) and portions of the 11S α-subunit were transformed into tobacco (Saalbach et al., 1991). Efficient vacuolar targeting was observed only when the entire α-chain was fused to CAT, suggesting that the targeting information was structural in nature.

Overall, the studies of vacuolar targeting of PHA, patatin and 11S globulin legumin have indicated that sorting information can reside in a region of amino acids on the surface of a protein or in some secondary or tertiary structural determinant.

Plant vacuolar proteins in yeast

The sorting of proteins to the plant cell vacuole has been shown to be mediated by a diverse collection of targeting signals contained in an amino-terminal propeptide, a carboxyl-terminal propeptide, or a portion of the mature protein. However, it was previously unclear if plant vacuolar targeting sequences could be recognized in yeast. As mentioned in the previous section, the analysis of PHA targeting has shown that it contains two distinct vacuolar targeting signals, one specific for yeast and another which is utilized in plants. Many other vacuolar proteins from plants and their sorting determinants have been studied in yeast.

The 11S legumin subunit is transported to the yeast vacuole (Saalbach et al., 1991). Gene fusions of yeast invertase with different legumin

propolypeptide segments were constructed and expressed in yeast. Various segments of the 11S subunit were able to deliver a portion of these fusion proteins to the yeast vacuole. However, the sorting efficiency was correlated with the increasing length, and only the complete legumin α-chain was able to redirect >90% of the chimera to the vacuole. In addition, it was shown that short C-terminal segments of the α-chain when fused to the C-terminus of invertase could redirected this fusion protein to the vacuole.

The transport of sporamin was also tested in yeast. When sporamin constructs containing the wild type NTPP and a mutant propeptide which causes secretion in plants, were expressed in yeast, both were delivered to the vacuole. The vacuolar targeting of the mutant NTPP sporamin suggests that a cryptic yeast targeting signal is contained within its sequence or structure (Matsuoka and Nakamura, 1992).

A more definitive study of a plant vacuolar targeting signal in yeast was conducted using the CTPP of BL. When this plant vacuolar signal was fused to the C-terminus of the yeast invertase and expressed in yeast, it was secreted from the cell (Gal and Raikhel, 1994), demonstrating that the CTPP of BL is not recognized in yeast. In addition, invertase-BL fusion proteins with or without the CTPP of BL were retained intracellularlly in yeast. Interestingly, when wheat germ agglutinin isoform 2 (WGA2), a homologue to BL sharing 94% amino acid identity (Wright, 1987; Wright et al., 1993; Lerner and Raikhel, 1989), was introduced into yeast with or without its CTPP, both proteins were secreted from the cell (Nagahora et al. 1992). The difference in sorting of BL and WGA2

may be due to the misfolding of the invertase-BL fusions, which were unable to bind to an *N*-acetyl glucosamine affinity column (Wilkins et al., 1990). In contrast, WGA2 displayed sugar binding activity. It should be noted that the ability of BL and WGA2 to bind *N*-acetyl glucosamine is used to determine if the proteins have folded and dimerized correctly (Wright, 1987; Wilkins et al., 1992; Wright et al., 1993). Therefore, the misfolding of the BL portion of the invertase-BL fusion protein may have exposed a cryptic yeast targeting signal, which mediated its intracellular retention. In addition, when two soluble plant vacuolar lectins from the legume *Dolichos biflorus*, seed lectin and DB58, were expressed in yeast, both were secreted from the cell (Chao and Etzler, 1994).

All these studies indicate that plants and yeast utilize different signals and/or mechanisms for protein transport to the vacuole. Thus, it appears that yeast is not an appropriate heterologous system in which to study the targeting of plant vacuolar proteins.

Targeting of membrane proteins

The majority of mammalian lysosomal membrane and soluble proteins are delivered to the lysosome via vesicular transport through an extensive endosomal system which includes the plasma membrane (PM). Membrane proteins are targeted to the lysosome by positive sorting information contained in their cytoplasmic tail (Peters et al., 1990; for review see Sandoval and Bakke, 1994). Mutations that alter the sorting determinant of lysosomal

membrane proteins, or disrupt the retention of ER and Golgi resident membrane proteins cause them to be localized to the PM (Jackson et al., 1990; Machamer and Rose, 1987; Williams and Fukuda, 1990; for reviews see Machamer, 1991; Pelham and Munro, 1993; Sandoval and Bakke, 1994), indicating that the default pathway for mammalian membrane proteins which enter the secretory pathway is the PM.

Yeast vacuolar proteins are sorted at the TGN and are transported by vesicles via a prevacuolar endosomal-like compartment (Vida et al., 1993). The resident vacuolar proteins dipeptidyl aminopeptidase (DPAP) B and alkaline phosphatase (ALP) were used to study yeast vacuolar membrane targeting. Both ALP (Klionsky and Emr, 1989) and DPAP B (Roberts et al., 1989) are type II membrane glycoproteins with a short N-terminal cytoplasmic domain, a single transmembrane domain, and a C-terminal catalytic lumenal domain. Extensive deletion and domain exchange studies were performed. As a result of these analyses, it was concluded that apart from being attached to the membrane, no structural or sequential information was necessary for these membrane proteins to reach the vacuole (Klionsky and Emr, 1990; Roberts et al., 1992).

In addition, when the cytoplasmic domains of the Golgi resident proteins DPAP Ap, Kex1p, and Kex2p were deleted, they were no longer retained in the Golgi, but were delivered to the vacuolar membrane (Cooper and Bussey, 1992; Roberts et al., 1992; Wilcox et al., 1992; for review, see Wilsbach and Payne, 1993a). These findings indicate that in yeast the default destination for secretory pathway membrane proteins is the vacuolar membrane (for reviews

see Stack and Emr, 1993; Nothwehr and Stevens, 1994). This is in direct contrast to the active sorting process for soluble vacuolar proteins, which require recognition of a targeting signal for delivery to the vacuole. This information, coupled with the fact that *vps* mutants do not cause vacuolar membrane proteins to be secreted to the cell surface (for review see Raymond et al., 1992b), indicates that there are several different mechanisms for yeast vacuolar protein sorting. However, more recent findings showed that in *vps1* mutant cells vacuolar membrane proteins are delivered to the vacuole by way of the PM (Nothwehr et al., 1995). The *VPS1* gene encodes a GTPase associated with the TGN which is necessary for proper sorting of soluble vacuolar proteins (Vater et al., 1992) and for the retention of Golgi membrane proteins (Wilsbach and Payne, 1993b).

In plants, the transport of membrane proteins is one of the least characterized aspects of vacuolar sorting. Progress in this area has been hampered in part by the lack of reporter proteins. The tonoplast intrinsic protein (TIP), a membrane protein of the aquaporin family (Chrispeels and Maurel, 1994), is synthezied on the rough ER before transport to the vacuolar membrane (Mäder and Chrispeels, 1984; Johnson et al., 1990; Höfte et al., 1991). The protein has six membrane spanning domains with cytoplasmically oriented N-terminal and C-terminal domains, and is present in different isoforms (Johnson et al., 1990; Höfte et al., 1992). The approach chosen to study vacuolar membrane protein targeting was to follow the fate of fusion proteins consisting of the chimeric secreted protein ssPAT (signal sequence -

phosphinotricine acetyltransferase) (Deneke et al., 1990) and the sixth transmembrane domain of ∝-TIP with or without its cytoplasmic domain (Höfte and Chrispeels, 1992). This analysis demonstrated that the sixth transmembrane domain alone was sufficient to transport the reporter protein to the vacuolar membrane. The delivery of this chimeric fusion protein to the tonoplast resembles the findings in yeast. Does this indicate that the default destination for secretory membrane proteins is to the vacuole? This question still needs to be clarified. If the default destination for vacuolar membrane proteins is found to be the vauole, then a more intriguing question arises; what is the necessary sorting information contained in secreted membrane proteins for transport to and retention at the PM? Interestingly, the G-protein from vesicular stomatitis virus (VSVG), which has a transmembrane domain, was transformed into tobacco and was delivered to the PM via the Golgi (Galbraith et al., 1992). Further study of the transport of this heterologous marker protein as compared with endogenous resident PM proteins is necessary to determine requirements for protein sorting to the PM.

Additional experiments were conducted, which compared the delivery of soluble PHA and vacuolar membrane ∝-TIP in the presence of the inhibitors monensin and BFA in tobacco (Gomez and Chrispeels, 1993). The analysis showed that PHA transport was inhibited, while the delivery of ∝-TIP to the tonoplast was not. This suggests that in plants, as in yeast, soluble and membrane proteins utilize different mechanisms for delivery to the vacuole.

Soluble plant vacuolar proteins utilize multiple mechanisms and receptors

Some plant cells contain more than one vacuole, which may perform different cellular functions. Since plant vacuolar proteins utilize more than one type of targeting determinant, would proteins utilizing different signals colocalize to the same vacuole? When sporamin (NTPP) and BL (CTPP) were expressed in the same tobacco plant, both were shown to be delivered to the same vacuoles in the cell (Schroeder et al., 1993). Recently, it was also shown that the CTPP of BL and the NTPP of sporamin are functionally interchangeable by the ability of either signal to target both BL and sporamin to the vacuole of tobacco BY-2 cells (Matsuoka et al., 1995).

Although proteins utilizing either NTPP and CTPP targeting signals are delivered to the same vacuoles, and the targeting signals are functionally interchangeable, there is now strong evidence of multiple receptors and mechanisms for the targeting of soluble proteins to the vacuole. While CTPPs have no consensus sequence, NTPPs display a common motif (see Figure 1.1), a specific substitution of the isoleucine with glycine inactivates the targeting signal and causes secretion. This specificity allowed for the identification of a putative vacuolar receptor for NTPP (Kirsh et al, 1994). This NTPP binding protein was isolated from extracts of developing pea cotyledons CCVs, based on its ability to bind to an affinity column containing the NTPP of proaleurain. This receptor protein is reported to be a integral membrane glycoprotein of 80 kDa with an N-terminal lumenal domain with a binding constant of 37 nM.

Binding assays demonstrated that NTPP peptides were able to compete for binding, whereas the mutant NTPP (glycine substituted for isoleucine) peptide and CTPP of BL could not. The inability of CTPP targeting signals to compete for binding indicates that there are multiple receptors involved in the targeting process.

In yeast, the *VPS34* gene encodes a phosphotidylinositol 3-kinase (PI 3-kinase), which has been shown to be necessary for the correct sorting of soluble vacuolar proteins (Herman and Emr, 1990; Shu et al., 1993; Stack et al., 1993). Recently, a specific inhibitor of mammalian PI 3-kinase, wortmannin (Nakanishi et al., 1992; Arcaro and Wymann, 1993; Yano et al., 1993; Thelen et al., 1994; Woscholski et al., 1994), was used in plants to investigate its effects on the delivery of NTPP and CTPP containing proteins to the vacuole (Matsuoka et al., 1995). Pulse chase analyses in tobacco BY-2 cells indicated that wortmannin at a concentration of 33 μM caused secretion of proteins utilizing CTPP targeting signals, whereas NTPP mediated transport of proteins to the vacuole displayed almost no sensitivity at this concentration of inhibitor. This differential sensitivity to wortmannin suggests two different mechanisms for sorting of soluble vacuolar proteins.

Components of plant vacuolar sorting machinery

In addition to the isolation of the putative NTPP receptor described earlier in this review, additional potential components of the plant vacuolar sorting

machinery have been identified. Recently, two small GTP binding proteins of 25 kDa and 27 kDa were isolated by biochemical means from vesicles targeted to the vacuoles in developing pumpkin cotyledons (Shimada et al., 1994). Small GTPases are believed to facilitate targeting of vesicles to their appropriate membranes (for reviews see Balch, 1990; Pryer et al., 1992; Novick and Brennwald, 1993; Ferro-Novick and Novek, 1994; Verma et al., 1994). The identification of two different small GTPases suggest that there may be mixed populations of vesicles destined for the vacuole. So far, only one GTPase in yeast has been identified that is involved in vacuolar targeting, the rab 5-like GTP-binding protein VPS21p (Horazdovsky et al., 1994). It will be interesting to determine if either of these GTPases will be able to complement the yeast *vps21* mutant.

A cDNA, *AtVPS34*, encoding for a PI 3-kinase was cloned by PCR from *Arabidopsis thaliana* (Welters et al., 1994). The protein sequence of *AtVPS34* shows homology to the yeast *VPS34p,* which has been shown to be necessary for the correct sorting of soluble vacuolar proteins (Herman and Emr, 1990). Despite the homology, the *AtVPS34* gene was unable to rescue the *vps34* deletion mutant. However, a chimeric gene in which the coding sequence for the C-terminal third of yeast *VPS34* (Catalytic domain) was replaced with the corresponding sequence from the plant gene was able to complement the mutation. The N-terminal domain of VPS34p is believed to mediate its interaction with the TGN and another protein kinase, VPS15p, in yeast (Stack et al., 1993). Therefore, some homologous plant proteins may be unable to

function in the yeast sorting apparatus because their sequences are divergent enough to disrupt the specificity of protein-protein interactions. Futhermore, the expression of *AtVPS34* antisense constructs resulted in plants that were severely inhibited in their growth and development.

Recently, the plant cDNA *aG68,* which encodes a large GTP-binding protein, was isolated from *Arabidopsis*. A sequence comparison shows it to have 53% sequence similarity at the amino acid level to the yeast *VPS1* gene (Dombrowski and Raikhel, 1995). In yeast, *vps1* mutants exhibit severe defects in soluble vacuolar protein sorting causing their mislocaliztion and secretion from the cell (Vater et al., 1992). The *VPS1* gene codes for a GTP-binding protein (Rothman et al., 1990) that is associated with the TGN, and is involved in retention of proteins in the Golgi (Wilsbach and Payne, 1993) as well as protein transport to the yeast vacuole (Vater et al., 1992; Nothwehr et al., 1995). However as with the *AtVPS34*, *aG68* was also unable to complement the yeast mutant. The greatest region of divergence between the plant and yeast proteins is also in a domain believed to be involved in protein interactions. The creation of a mutant phenotype by suppressing the expression of the *aG68* gene by antisense in plants or by a construct of a dominant mutation (Vater et al., 1992) are currently in progress in an attempt to identify its function.

A third plant cDNA homologue of yeast *vps* mutants has been isolated by functional complementation of the yeast *pep12* mutant (Jones, 1977). The yeast *PEP12* gene is necessary for the delivery of soluble proteins to the vacuole. Yeast PEP12p is a member of the syntaxin family of intergal membrane

proteins which are believed to function as receptors for transport vesicles (Bennett et al., 1993; Calakos et al., 1994; for reviews, see Ferro-Novick and Jahn, 1994; Rothman, 1994). The *Arabidopsis* cDNA (*aPEP12*) potentially encodes for a 31 kDa protein which has homology to other members of the syntaxin family, in addition to yeast PEP12 (Bassham et al., 1995).

The future

The main focus of current research is to identify and isolate components of the vacuolar sorting machinery and to elucidate their role in transport to the vacuole. The isolation of a receptor to the CTPP by biochemical methods has been unsuccessful. In contrast to the specific motif present in the NTPP of yeast CPY and barley aleurain, research has shown that the CTPP has no consensus sequence. In addition, the component of the sorting machinery that interacts with the CTPP binds to very short stretches of amino acids. The putative receptor may also have a low binding affinity for the CTPP, thereby making it extremely difficult to isolate by biochemical means.

In yeast, the creation of the vacuolar protein sorting (*vps*) mutants (for reviews, see Klionsky et al., 1990; Raymond et al., 1992b) have played an essential role in the isolation and identification of components as well as the elucidation of mechanisms involved in the vacuolar sorting process. The plant *Arabidopsis thaliana* has many characteristics which make it a very good model system for the generation of mutants (Estelle and Somerville, 1986; Somerville,

1989; Koncz and Rèdei, 1994). We have recently shown that when BL is transformed into *Arabidopsis* it is correctly processed and targeted to the vacuoles in roots and leaves (J.E. Dombrowski, O. Borkhsenious, A. Sandul, and N.V. Raikhel unpublished results). Therefore, the creation of vacuolar protein sorting mutants in *Arabidopsis* (*avps*) will provide an excellent chance to isolate the CTPP receptor and other components of the sorting apparatus. One of the drawbacks of creating *avps* mutants is that mutations in vacuolar protein sorting may severely disrupt the structure or inhibit the formation of the vacuole, the presence of which is believed to be essential for plant cell growth. However, most of the yeast *vps* mutants are not lethal. In addition, the yeast *vps* mutants display a variety of vacuolar morphologies (Klionsky et al., 1990; Raymond et al., 1992 a,b). Furthermore, by using ethylmethane sulfonate mutagenesis, one can potentially create conditional (temperature sensitive) or leaky mutants which will allow for the isolation of essential genes (Feldmann et al., 1994). One of the requirements for the development of a good genetic screen is a genetic marker or reporter protein, which yields a phenotype that is quick and easy to score. At the 1995 Keystone meeting on Plant Cell Biology, Dr. Jean-Marc Neuhaus reported that when the seven amino acid CTPP of tobacco chitinase was fused to the C-terminus of rat beta-glucuronidase (RGUS+T), it was efficiently targeted to the vacuole in tobacco (Chrispeels et al., 1995). Therefore, one can envision a screen for *avps* mutants, whereby a colorimetric assay will be used to select for seedlings whose roots are missorting and secreting the vacuolar localized RGUS+T.

Mutants and cell-free assay/transport systems coupled with biochemical approaches have allowed for the identification of components involved in the secretory pathway (ER-Golgi-Lysosome/Vacuole) in mammalian and yeast systems (for reviews, see Klionsky et al., 1990; Rothman and Orci, 1992; Pryer et al., 1992; Raymond et al., 1992b; Rothman, 1994) and have led to the elucidation of the mechanisms involved in protein transport through the endomembrane system.

In this review I have indicated that plants are fundamentally different compared to mammalian and yeast systems in the way soluble vacuolar proteins are targeted to the vacuole. Currently, little is known about the mechanisms or machinery involved in targeting proteins to the plant cell vacuole. Some of the components and clues to mechanisms involved in vacuolar transport have been discussed in this review. The future for vacuolar targeting in plants will be led by the development of an in vitro transport system, combined with the generation of vacuolar sorting mutants in *Arabidopsis*. This more than anything else will finally answer the question: "How are proteins targeted to the plant vacuole?".

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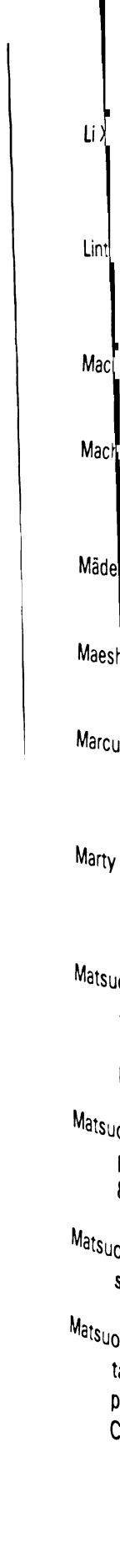
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CHAPTER 2

DETERMINATION OF THE FUNCTIONAL ELEMENTS WITHIN THE VACUOLAR TARGETING SIGNAL OF BARLEY LECTIN

Reference: Dombrowski JE, Schroeder MR, Bednarek SY, Raikhel NV (1993). Plant Cell **5**, 587-596.

ABSTRACT

We have previously demonstrated that the carboxyl-terminal propeptide of barley lectin is both necessary and sufficient for protein sorting to the plant vacuole. Specific mutations were constructed to determine which amino acid residues or secondary structural determinants of the carboxyl-terminal propeptide affect proper protein sorting. We have found that no consensus sequence or common structural determinants are required for proper sorting of barley lectin to the vacuole. However, our analysis demonstrated the importance of hydrophobic residues in vacuolar targeting. In addition, at least three exposed amino acid residues are necessary for efficient sorting. Sorting was disrupted by the addition of two glycine residues at the carboxyl-terminal end of the targeting signal or by the translocation of the glycan to the carboxy terminus of the propeptide. These results suggest that some components of the sorting apparatus interact with the carboxy terminus of the propeptide.

INTRODUCTION

The eukaryotic cell is organized into distinct, specialized membrane-bound subcellular compartments, each characterized by its own defined subset of proteins. Delivery to and retention of these proteins within their specialized compartments is dependent upon specific targeting information present in the sequence, structure, and/or post-translational modifications of the protein.

Proteins found in the endoplasmic reticulum (ER), Golgi apparatus, lysosomes, vacuoles/protein bodies, plasma membrane, and cell wall are derived from a subset of proteins that enter the secretory pathway. The vast majority of these proteins have an amino-terminal hydrophobic signal sequence that mediates membrane translocation from the cytosol to the lumen of the ER (von Heijne, 1988). Secretory proteins may undergo further processing in the ER and Golgi network (for review, see Chrispeels, 1991). Retention and sorting within the secretory pathway, however, requires additional targeting information. Proteins lacking this information follow a default pathway and are secreted to the cell surface (for review, see Bednarek and Raikhel, 1992).

The best characterized targeting signal is the mannose-6-phosphate residue that specifies transport of hydrolytic enzymes to the mammalian lysosome (Kornfeld and Mellman, 1989). In yeast, two vacuolar proteins, carboxypeptidase Y and proteinase A, contain sorting information within an amino-terminal propeptide (Johnson et al., 1987; Valls et al., 1987; Klionsky et al., 1988). A detailed mutational analysis of the carboxypeptidase Y

propeptide determined that the tetrapeptide QRPL is critical for sorting of the protein to the vacuole (Valls et al., 1990); however, the amino-terminal propeptide of the hydrolase proteinase A shares no significant similarity with the CPY sorting domain (Klionsky et al., 1988). Currently, no consensus sequence or structural determinant has been identified for vacuolar targeting in yeast, which indicates that a diverse array of factors are involved in the sorting process.

Vacuolar targeting in plants can be mediated by targeting signals contained in an amino-terminal propeptide, a carboxyl-terminal propeptide, or a mature portion of the protein (for reviews, see Bednarek and Raikhel, 1992, Chrispeels and Raikhel, 1992). The vacuolar storage protein sporamin from sweet potato (Matsuoka and Nakamura, 1991) and the vacuolar thiol protease aleurain from barley (Holwerda et al., 1992) contain their targeting information within an amino-terminal propeptide. A comparison of the deduced amino acid sequences of these amino-terminal propeptides and other known vacuolar proteins with amino-terminal extensions shows that they do share a common motif (NPIRL\P) within their sequences (for reviews, see Chrispeels and Raikhel, 1992; Bednarek and Raikhel, 1992). This motif is critical for proper sorting (Nakamura and Matsuoka, 1993), since a glycine substitution for the conserved isoleucine or asparagine residues in the targeting sequence of sporamin results in the secretion of prosporamin from the cell. In contrast, the of sorting information for the vacuolar proteins, phytohemagglutinin, 11S legumin, and patatin have been shown to be contained within portions of the mature protein

(for a review, see Bednarek and Raikhel, 1992). However, these protein regions share no sequence identity. Many vacuolar proteins also have carboxyl-terminal propeptides (CTPPs) that share no common sequence identity but have short stretches of hydrophobic amino acids (Bednarek and Raikhel, 1992).

Our investigation has focused upon the Gramineae barley lectin (BL), a homodimeric vacuolar protein that specifically binds the sugar *N*-acetylglucosamine (Figure 2.1A) (for review, see Raikhel and Lerner, 1991). BL is initially synthesized as a preproprotein with a high-mannose glycosylated CTPP that is removed before or concomitant with deposition of the mature protein into the vacuole, as shown in Figure 2.1B. The CTPP is a hydrophobic 15-amino acid peptide that contains 2 acidic residues (Figure 2.1C) and has the potential to form an amphipathic α-helix. It has been demonstrated that this sequence is necessary for proper sorting of BL to the plant vacuole (Bednarek et al., 1990) and is sufficient to redirect a normally secreted protein, cucumber chitinase, to the vacuole of transgenic tobacco plants (Bednarek and Raikhel, 1991). Therefore, the BL CTPP contains vacuolar targeting information within its sequence.

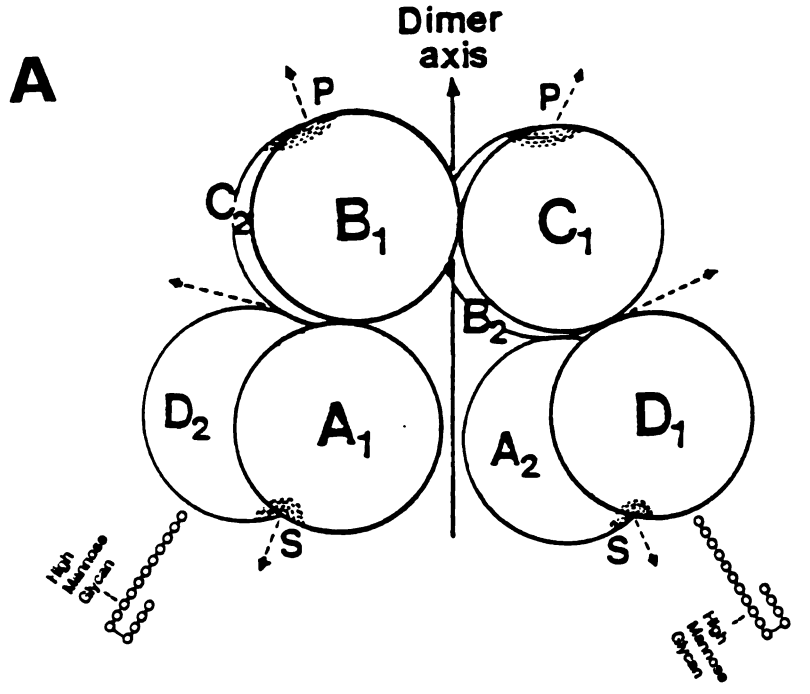
In this study, we extended the analysis of the CTPP to identify and define the essential features for vacuolar protein targeting. We have used both transgenic and transient expression systems to define the minimum requirements for proper sorting of BL to the plant vacuole.

Figure 2.1. Schematic Representation of proBL, its Maturation in the Secretory Pathway and the Carboxyl-Terminal Propeptide Amino Acid Sequence.

(A) Probarley lectin is a homodimeric protein, each subunit consisting of four homologous domains of 43 amino acids each, which come together in a reversed orientation to form primary (P) and secondary (S) sugar binding sites and a 15-amino acid carboxyl-terminal propeptide containing an N-linked high-mannose glycan. (Wright, 1987: Wright et al., 1993).

(B) The preproprotein of barley lectin gains access to the secretory pathway by a 26-amino acid signal sequence which is cotranslationally removed. In the lumen of the ER, the 23-kD subunits of the proprotein dimerize to form an active sugar-binding lectin. The dimerized proprotein moves through the Golgi apparatus and is transported to the vacuole. Prior to or concomitant with deposition into the vacuole, the glycosylated CTPPs are cleaved off to yield the mature lectin consisting of two identical 18-kD subunits.

(C) The 15-amino acid CTPP of BL (amino acids at positions 172 to 186). Also depicted is the N-linked high-mannose glycosylation attachment site at amino acid position 180.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P
C2
B1
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B2
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S
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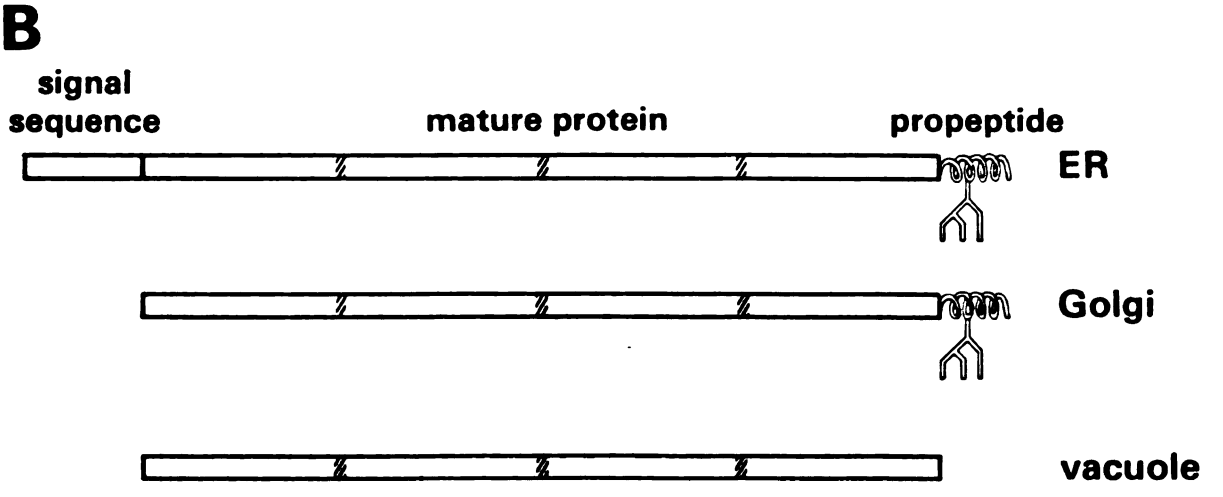
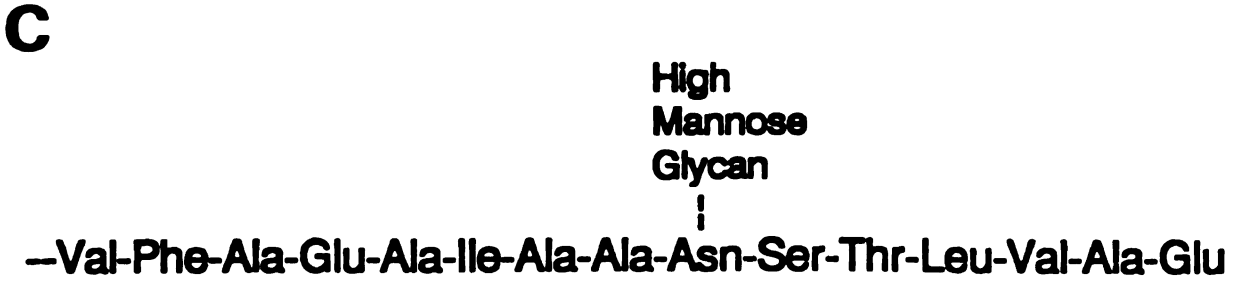
High Mannose Glycan
High Mannose Glycan


B
signal sequence
mature protein
propeptide
ER
Golgi
vacuole


C
High Mannose Glycan
--Val-Phe-Ala-Glu-Ala-Ile-Ala-Ala-Asn-Ser-Thr-Leu-Val-Ala-Glu

RESULTS

Construction of the Mutant CTPPs of Barley Lectin

Several BL cDNA clones containing mutant CTPPs were prepared using site-specific mutagenesis of the CTPP coding region, to identify and define the essential features in the CTPP that are necessary for vacuolar protein targeting. Figure 2.2 describes and summarizes the intracellular and extracellular distribution of the BL-mutant CTPP proteins expressed in stably and transiently transformed tobacco leaf cells.

Localization of BL-mutant CTPP Constructs 1 (VNSTLVAE), 2 (VFAEAIAA),and 3 (VFAEAI) in Transgenic Plants

To determine the specific regions of the CTPP necessary to target BL + CTPP (wild type) to the vacuole, we designed deletion mutants 1 (VNSTLVAE), 2 (VFAEAIAA) and 3 (VFAEAI) (Figure 2.2). Transgenic tobacco plants expressing the BL-mutant CTPP constructs (1, 2 and 3) were obtained via *Agrobacterium*-mediated transformation of tobacco. Subcellular localization of BL in transgenic tobacco plants, by electron microscopic (EM) immunocytochemistry localized BL deletion mutant 3 (VFAEAI) to the vacuole, as shown in Figure 2.3A. EM immunocytochemical analysis of transgenic plants expressing mutants 1 (VNSTLVAE) and 2 (VFAEAIAA) also showed specific localization to the

Figure 2.2. Description and Summary of the Intracellular and Extracellular Distribution of BL-CTPP Mutants.

Each BL-mutant CTPP construct is represented by their sequence, using the single letter amino acid codes and divided according to its intracellular or extracellular distribution. The code CDG- refers to the last three amino acids in the mature protein, of which the C residue is involved in the formation of intramolecular disulfide bond (Wright, 1987). Dots represent the deletion of amino acids from the wild-type (WT) construct. Outlined letters represent amino acid substitutions, insertions, or glycine replacements of existing CTPPs. Constructs designated by asterisks represent those mutants analyzed in transgenic plants. Based on scanning densitometry, BL-mutant CTPP constructs designated as intracellular are retained $\geq$ 95%, except construct 27 which is $\geq$ 90%. BL-mutant CTPP constructs designated as extracellular are those constructs that show the same pattern of secretion as control construct 10 (total deletion of CTPP), that is, 95% secreted.

INTRACELLULAR	EXTRACELLULAR

WT* CDG - VFAEAIAANSTLVAE

DELETION ANALYSIS

01.* CDG - V.....NSTLVAE	**09. CDG - VF.....**
02.* CDG - VFAEAIAA.....	**10.* CDG - .....**
03.* CDG - VFAEAI.....	
04. CDG - VFA.....NST....	
05. CDG - VFA.....GST....	
06. CDG - VFAEA.....	
07. CDG - VFAE.....	
08. CDG - VFA.....	
11. CDG - V.....STLVAE	
12. CDG - .....LVAE	

GLYCINE SUBSTITUTION AND GLYCOSYLATION SITE SHIFT ANALYSIS

13. CDG - VFAEAG	**15. CDG - VFAEGG**
14. CDG - VGAEAG	**16. CDG - VFAGGG**
19. CDG - VFAG	**17. CDG - VFGGGG**
20. CDG - VFG	**18. CDG - VGGGGG**
	21. CDG - VFAEAIAANSTLVAEGG
	22. CDG - VFAEAIAAGSTLVNATE

ARTIFICIAL CTPPs

23. CDG - AVIDVA	**28. CDG - EEEE**
24. CDG - AVIAVA	**29. CDG - KKKK**
25. CDG - AAAA	
26. CDG - VFAEAD	
27. CDG - KDAEAD	
30. CDG - LLVD	
31. CDG - PIRP	

32. CDG - KMQRQTDDLANGSIIATTNNPWQFCCHVRSPFTYF

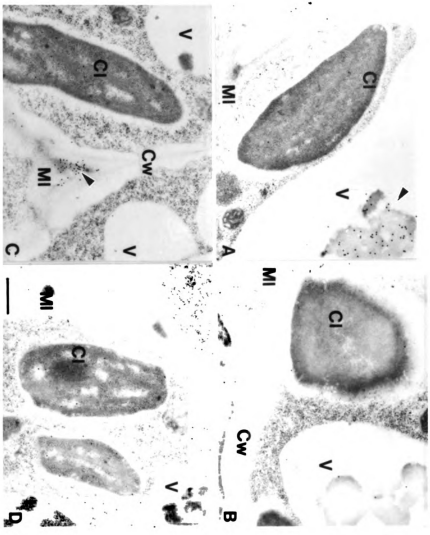
ADDITIONAL MUTATIONAL STUDIES

33.* CDG - VFAEPIPANSTLVAE	
34.* CDY - VFAEAIAANSTLVAE	
35.* CDY - WFAEAIAANSTLVAE	
36.* CDG - VFAQAIAANSTLVAQ	
37. CDG - VFAKAIAANSTLVAK	

Figure 2.3. Immunocytochemical Localization of BL-mutant CTPP constructs 3 and 10.

(A) and **(C)** Thin sections of transgenic tobacco leaves expressing BL-mutant CTPP constructs 3 (VFAEAI) **(A)** and 10 (total deletion of CTPP) **(C)** treated with rabbit polyclonal anti-WGA antisera.

(B) and **(D)** Thin sections of transgenic tobacco leaves expressing BL-mutant CTPP constructs 3 (VFAEAI) **(B)** and 10 (total deletion of CTPP) **(D)** treated with nonimmune sera. Gold labeling (arrow) is found exclusively in the vacuole of tobacco plants transformed with BL-mutant CTPP construct 3 (VFAEAI) **(A)** and within the middle lamella of transgenic BL-mutant CTPP construct 10 (total deletion of CTPP) **(C)**. Bar = 0.5 μm. Cw, cell wall; V, vacuole; MI, middle lamella; Cl, chloroplast.


A
Cl
MI
V
B
Cw
C
D

vacuole (data not shown). We have previously shown by pulse-chase analysis that tobacco plants expressing BL-mutant CTPP construct 10 (total deletion of the CTPP) secreted BL, yet a small amount of the protein remained associated with the protoplasts (Bednarek et al., 1990). However, as shown in Figure 2.3C, the EM immunocytochemical analysis of tobacco plants expressing BL deletion mutant 10 showed no detectable labeling in the vacuoles but was localized to the middle lamella of tobacco leaf cells. No specific labeling was detected in parallel experiments using nonimmmune serum (Figures 2.3B and 2.3D).

The subcellular distribution of BL-mutant CTPP constructs was also confirmed by organelle fractionation. Vacuoles were isolated from protoplasts of transgenic plants that expressed BL-mutant CTPP construct 3 (VFAEAI) . BL was affinity purified from protein extracts isolated from protoplast and vacuolar fractions containing equal amounts of α-mannosidase activity (a vacuolar-specific marker enzyme) and examined by SDS-PAGE and immunoblot analysis. As shown in Figure 2.4A, the 18-kD subunit for mature BL was present at similar levels in the protoplast and vacuolar fractions of plants expressing CTPP mutant construct 3. ER contamination of the vacuolar fractions was minimal, since they contained $\leq$10% NADH-cytochrome *c* reductase relative to total protoplast-associated activity.

Figure 2.4. Subcellular Localization and Pulse-Chase Labeling Experiments of Transgenic and Transient Protoplasts Expressing BL-Mutant CTPP Construct 3.

(A) Immunoblot analysis of affinity-purified BL from protoplasts and vacuoles isolated from transgenic tobacco plants expressing BL-mutant CTPP construct 3 (VFAEAI). Protoplast and vacuole fractions containing equal amounts of *α*-mannosidase activity were loaded per lane.

(B) Pulse-chase labeling of protoplasts isolated from transgenic tobacco plants expressing BL-mutant CTPP construct 3 (VFAEAI) and corresponding incubation medium. Protoplasts were pulse labeled for 4 hr and chased for 18 hr.

(C) Pulse-chase labeling of protoplasts transiently expressing the BL-mutant CTPP construct 3 (VFAEAI) and corresponding incubation medium. Protoplasts were pulsed for 8 hr and chased for 12 hr.

Protein extracts were prepared from the protoplasts and incubation media at specified time intervals (hr) as indicated during the chase. Radiolabeled BL was affinity purified and analyzed by SDS-PAGE and fluorography. The molecular mass of the mature 18-kD subunit of BL is shown to the left of the gels.

A
vacuole
protoplast
B
Chase time (hr) 0 3 6 9 12 15 18
Protoplasts
18kD –
Incubation media
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Comparison of Transient and Transgenic Expression Systems

Although we have previously demonstrated the reproducibility of transient assay data as it compared with the findings obtained from the analysis of transgenic plants (Bednarek et al., 1990; Bednarek and Raikhel, 1991), the initial set of BL-mutant CTPP constructs were analyzed using both transient and transgenic systems, to insure that no variability existed in short deletion mutants. Processing and targeting of BL+CTPP (wild type) and BL-mutant CTPP construct 10 (total deletion of CTPP), were previously characterized and described in Bednarek et al. (1990), and were used as positive (targeting to the vacuole) and negative (secretion of BL) controls in every analysis performed with the BL-mutant CTPP constructs.

The BL-mutant CTPP constructs were examined by pulse and pulse-chase analyses. Protoplasts from transgenic plants were pulse-labeled for 2-4 hr in the presence of a mixture of ^{35}S-labeled methionine and cysteine and chased for an additional 18 hr in the presence of excess unlabeled methionine and cysteine. In Figure 2.4B, the deletion mutant 3 (VFAEAI) showed that radiolabeled BL was readily discernible with 4 hr of pulse-labeling. After an 18-hr chase period, no detectable secretion of the BL-mutant CTPP to the incubation medium was observed. During the course of the chase, the level of labeled BL protein remained constant, demonstrating the stability of BL in the vacuole over the time interval. This result was confirmed by continuous labeling of protoplasts from mutant 3 (VFAEAI) for 18 hr, and no detectable

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radiolabeled BL was observed in the incubation medium, while the accumulation of BL intracellularly increased steadily over the same time period (data not shown).

Rapid analysis of the BL-mutant CTPP constructs was performed by transient expression in tobacco leaf protoplasts. The data from transient expression presented in Figure 2.4C for BL deletion mutant 3 (VFAEAI) correlated directly with the findings obtained in transgenic plants, whereas no radiolabeled BL was detected in the incubation medium, and an 18-kD band was retained intracellularly. The same results were obtained for mutant 1 (VNSTLVAE) and 2 (VFAEAIAA); both were retained intracellularly with no detectable secretion to the incubation media (data not shown). Therefore, the reproducibility of the results obtained from the analysis of transgenic plants compared with that from transient expression was demonstrated, and the transient system was utilized for further analysis of the remaining BL-mutant CTPP constructs.

Deletion Analysis of BL-mutant CTPP Constructs

Deletion analysis of the CTPP described above identified two independent regions of the 15-amino acid propeptide, each necessary for proper sorting of BL to the vacuole in transgenic tobacco. Comparison of CTPP deletion mutants 1 (VNSTLVAE) and 3 (VFAEAI) revealed no readily apparent consensus sequence. However, the sequence of each CTPP deletion mutant contained

similar 4-amino acid stretches, AEAI and LVAE, that may function as vacuolar targeting determinants. To address whether or not these regions were necessary for proper sorting, we designed deletion mutants 4 (VFANST) and 5 (VFAGST), in which these regions had been eliminated. The transient analysis of mutants 4 and 5 showed that radiolabeled BL was retained intracellularly, with no detectable accumulation of BL in the incubation media (data not shown). These results indicated that a more comprehensive mutational analysis of CTPP would be necessary to determine the nature of the sorting signal.

Determination of Minimum Length Required for Efficient Sorting of BL

Mutant 3 (VFAEAI) was chosen for a detailed deletional analysis, to determine the minimum length necessary for proper sorting of BL to the vacuole. Results of the transient analyses for the deletion series BL mutants 7 (VFAE), 8 (VFA), 9 (VF) and 10 (total deletion of CTPP) are shown in Figure 2.5A. Constructs 7 and 8 were retained intracellularly over the course of the chase, with no significant accumulation of BL in the media. A very faint 18-kD polypeptide was observed in the incubation medium from protoplasts expressing construct 8 after 12 hr of chase. The low-level missorting of mutant 8 may be due to high expression levels or to a small amount of cell lysis as compared with the positive control. Similarly, deletion mutant 6 (VFAEA) was retained intracellularly, with no detectable secretion into the medium (data not shown). As shown in Figure 2.5A, however, BL was secreted into the incubation media

Figure 2.5. Deletion and Glycine Replacement Analysis.

(A) Pulse-chase labeling of protoplasts transiently expressing the deletion BL-mutant CTPP constructs 7 (VFAE), 8 (VFA), 9 (VF), and 10 (total deletion of CTPP) and corresponding incubation media.

(B) Pulse-chase labeling of protoplasts transiently expressing the glycine replacement BL-mutant CTPP constructs 14 (VGAEAG), 13 (VFAEAG), 15 (VFAEGG), and 16 (VFAGGG) and corresponding incubation media.

Protoplasts were pulsed for 8 hr, chased for 12 hr, and analyzed as previously described. The molecular mass of the mature 18-kD subunit of BL is shown to the left of the gels.


A
construct
#7
#8
#9
#10
chase time (hr)
0 12 0 12 0 12 0 12
Protoplasts
18kD–
Incubation medium
18kD–
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#13
#15
#16
0 12 0 12 0 12 0 12
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of protoplasts expressing construct 9 and control (secretion) construct 10 and displayed increased accumulation of the 18-kD subunit over the course of the chase. Yet, after 12 hr of chase a small amount of 18-kD polypeptide remained associated with the protoplast fraction for both constructs. Retention of the 18-kD polypeptide may result from a continued low-level incorporation of labeled amino acids into a newly synthesized polypeptide as indicated by the presence of the 23-kD proprotein of BL-CTPP (wild-type, control), an association of BL with plasma membrane/cell wall remnants, or a low level of sorting to the vacuole. However, as shown in Figure 2.3C, the EM immunocytochemical analysis of transgenic plants expressing CTPP deletion construct 10 did not detect BL in the vacuole, but localized it to the middle lamella. We have also noted a decrease in signal intensity over time for media fractions, but not for those constructs retained intracellularly. This loss of signal is most likely due to protein absorption by the polystyrene tissue culture plates used to incubate the protoplasts during labeling and not due to degradation (data not shown).

Additional deletion mutants of 11 (VSTLVAE) and 12 (LVAE) were constructed to analyze whether or not the carboxyl-terminal region of the propeptide had the capacity to properly sort BL to the vacuole. After 20 hr of continuous pulse-labeling of protoplasts expressing deletion mutants 11 and 12, no radiolabeled BL was found in the incubation media, whereas an 18-kD polypeptide was retained intracellularly (data not shown).

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Glycine Replacement Analysis of Deletion Mutants Retained Intracellularly

A glycine replacement analysis of the vacuolar localized deletion mutant 3 (VFAEAI) was conducted to define specific residues involved in sorting BL to the vacuole. These constructs maintained the same length of the CTPP (six amino acids), while eliminating possible side-chain interactions. Pulse-chase labeling revealed that a minimum of two tandem glycine residues at the carboxyl-terminal end of the propeptide could disrupt proper sorting of BL to the vacuole. As shown in Figure 2.5B, constructs 15 (VFAEGG) and 16 (VFAGGG) resulted in significant accumulation of an 18-kD polypeptide in the incubation media after a 12-hr chase. After 12 hr, a portion of the radiolabeled protein remained associated with the protoplast at a level similar to that displayed by control construct 10 (total deletion of CTPP) (Figure 2.5A). Constructs 17 (VFGGGG) and 18 (VGGGGG) displayed patterns of secretion similar to those of constructs 15 (VFAEGG) and 16 (VFAGGG) (data not shown), while mutant CTPPs with a single glycine residue (construct 13, [VFAEAG]) or two glycine residues not in tandem (mutant 14, [VGAEAG]) remained intracellular for the same time interval (Figure 2.5B). Additional glycine replacement constructs (19, [VFAG], and 20, [VFG]) were designed to investigate whether the carboxyl-terminal residues of deletion mutants 7 (VFAE) and 8 (VFA) were necessary for proper sorting of BL. Constructs 19 and 20 remained intracellular and showed no accumulation of an 18-kD polypeptide in the incubation media (data not shown).

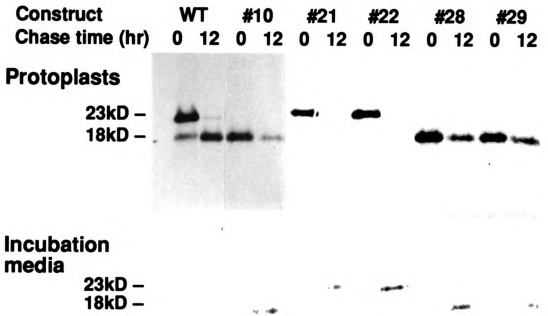
Tandem Glycine Residues or Glycan Shift to the Carboxy terminus of the CTPP Disrupts Sorting of BL

Comparison of the results obtained from the deletion and glycine replacement analyses revealed that although deletion mutant 7 (VFAE) was retained intracellularly, the addition of two glycine residues at the carboxy terminus (mutant 15, VFAEGG) caused it to be secreted (Figure 2.5B). Therefore, mutant 21 (VFAEAIAANSTLVAEGG) was constructed to analyze whether or not the addition of two glycine residues at the carboxyl-terminal end of the 15-amino acid wild-type propeptide could similarly disrupt proper sorting of BL to the vacuole. As shown in Figure 2.6, this resulted in the appearance and accumulation of a 23-kD proprotein in the incubation medium of protoplasts transiently expressing construct 21. After 12 hr of chase, no detectable 18-kD polypeptide (mature BL) was observed in the protoplast fraction. However, a very low level of the 23-kD proprotein still remained associated with the protoplast fraction.

These results suggested that the protein sorting information contained within the CTPP is blocked or inaccessable in mutants 15 to 17 and 21 to the sorting apparatus. To confirm this hypothesis, we designed mutant 22 (VFAEAIAAGSTLVNATE) in which the site of glycan addition was shifted close to the carboxy terminus of the propeptide. As shown in Figure 2.6, radiolabeled 23-kD proprotein was present in both the protoplasts and medium at the 0 hr chase time point, but there was an increased level of accumulation

Figure 2.6. Disruption of Proper Sorting of BL by Carboxyl-Terminal Tandem Glycine Residues, Glycosylation Site Shift, and Artificial Propeptides.

Pulse-chase labeling of protoplasts transiently expressing the *WT* (wild type) BL, BL-mutant CTPP constructs 10 (total deletion of CTPP), 21 (VFAEAIAANSTLVAEGG), 22 (VFAEAIAAGSTLVNATE), 28 (EEEE), and 29 (KKKK), and corresponding incubation media. Protoplasts were pulsed for 8 hr, chased for 12 hr and analyzed as previously described. The molecular masses of the mature 18-kD subunit and the wild-type 23-kD proprotein of BL are shown to the left of the gels.


Construct
WT
#10
#21
#22
#28
#29
Chase time (hr)
0 12 0 12 0 12 0 12 0 12 0 12
Protoplasts
23kD –
18kD –
Incubation media
23kD –
18kD –

of proprotein in the medium, with a concurrent decrease in the level of proprotein associated with the protoplasts after 12 hr of chase. The inhibition of glycosylation by tunicamycin, however, showed that the unglycosylated mutant was properly processed and sorted (data not shown). Thus, the presence of the glycan was responsible for secretion of proBL, and not the altered sequence at the carboxy terminus of mutant 22.

Analysis of Artificial CTPPs

Although no obvious consensus sequence exists among plant vacuolar targeting signals, a common feature among them is that they are rich in hydrophobic residues (for reviews, see Bednarek and Raikhel, 1992; Chrispeels and Raikhel, 1992). Artificial CTPPs, such as mutants 23 (AVIDVA), 24 (AVIAVA), and 25 (AAAA), were designed to analyze whether or not other short hydrophobic peptides could redirect BL to the vacuole, whereas mutants 26 (VFAEAD), 27 (KDAEAD), 28 (EEEE), and 29 (KKKK) were made to investigate the effect of nested charged residues upon sorting. Mutant 30 (LLVD) is homologous to a hydrophobic stretch from the vacuolar targeting CTPP of tobacco chitinase (Neuhaus et al., 1991), and mutant 31 (PIRP) represents a common motif present in amino-terminal propeptides of some vacuolar proteins (Chrispeels and Raikhel, 1992). Mutant 32 (KMQRQTDDLANGSIIATTNNPWQFCCHVRSPFTYF) is a random sequence of 35 amino acids which was generated as an artifact during the mutagenesis

process. BL-mutant CTPP constructs 23 to 27 and 30-32 were retained intracellularly after pulse-chase labeling (data summarized in Figure 2). In addition, BL-mutant construct 32 was not only retained intracellularly, but was processed down to roughly 18-kD (mature BL) from a 22.5-kD proprotein as measured by band mobility shift on SDS-PAGE (data not shown). However, construct 27 (KDAEAD) did show a very low level of secretion (5-10%) as compared to the controls (data not shown). Constructs 28 (EEEE) and 29 (KKKK) exhibited the same pattern of secretion into the incubation medium as control CTPP deletion mutant 10 (Figure 2.6).

Additional BL CTPP Mutants Addressing Structure and Processing.

Mutant 33 (VFAEPIPANSTLVAE) was designed to disrupt the predicted amphipathic α-helix by the exchange of proline residues for alanine residues within the CTPP, without changing its length. The analysis of mutant 33 in transgenic plants indicated that the predicted amphipathic α-helix did not appear to have any significant affect on the CTPP processing or on the proper sorting of BL to the vacuole. BL-mutant CTPP construct 36 (VFAQAIAANSTLVAQ) reduced the acidic character of the propeptide by conservative replacement of the glutamic acid residues with glutamine residues, while maintaining the secondary structure of the propeptide. BL-mutant CTPP construct 37 (VFAKAIAANSTLVAK) exchanged basic lysine residues for the glutamic acid residues. Neither construct affected the proper processing or sorting of BL to

the vacuole, however, construct 37 was not glycosylated (data not shown).

BL-mutant CTPP constructs 34 (CDY-VFAEAIAANSTLVAE) and 35 (CDY-WFAEAIAANSTLVAE) were designed to disrupt CTPP processing by increasing steric hindrance through modification of amino acid residues on both sides of the putative cleavage site (Wright, 1987). Neither of these constructs appeared to have any significant effect on CTPP processing or on the proper sorting of BL to the vacuole. However, BL deletion mutant 1 (VNSTLVAE) exhibited significant reduction of CTPP processing without disrupting the proper sorting of BL to the vacuole. Processing of the CTPP was first observed 20 hours after the addition of label as compared to 2-3 hours for the wild type BL control (data not shown).

DISCUSSION

Post-translational Processing of the Carboxyl-terminal Propeptide and its Effect on Vacuolar Targeting

Little is known about the mechanisms involved in the post-translational processing of propeptides of plant vacuolar proteins (Chrispeels, 1991). The processing of eukaryotic signal sequences as well as secretory signal sequences in prokaryotes is dependent on sequence-specific endopeptidases (von Heijne, 1988). In *Escherichia coli*, proper cleavage of the secretory sequence can be disrupted by increasing the size of the amino acid side chains at the cleavage site (Pollitt et al., 1986). The carboxyl-terminal glycine residue is conserved in all mature cereal lectins (for review, see Raikhel and Lerner, 1991). Although the actual cleavage site for all these proteins is not known, X-ray crystallographic analysis and carboxyl-terminal sequencing has shown that mature wheat germ agglutinin (WGA), a homolog of BL, ends with a glycine residue (Wright, 1987). In our study, the addition of large side chains at the junction of the mature protein and the CTPP (mutant constructs 34 and 35), did not block processing or sorting. The result may simply mean that the increase in size of the side chains from glycine and valine to tyrosine and tryptophan residues is not sufficient to block processing, or an alternative cleavage site is utilized. However, pulse-chase labeling of protoplasts expressing BL deletion mutant #1 (-VNSTLVAE), in which the glycosylation site and putative cleavage

site were maintained, showed a significant decrease in conversion of the proprotein into the mature form as compared to the BL+CTPP (wild type) construct (data not shown). The decrease in processing, however, did not disrupt proper sorting of BL to the vacuole. The mutant proprotein migrated as a 21.8-kD protein by SDS-PAGE, indicating that the mutant CTPP peptide was glycosylated properly. The proximity of the bulky high mannose residue may cause a drastic conformational change at the cleavage site or may present steric hindrance to an endopeptidase, thereby disrupting processing. In addition, deletion mutant 1 (VNSTLVAE) may have eliminated the portion of the propeptide which may contain the cleavage site.

Further analysis of the deleted sequence of mutant 1 revealed that it contained an *E. coli* cleavage site motif Ala-X-Ala (Perlman and Halverson, 1983). However, mutant construct 33, originally designed to disrupt the secondary structure by substituting proline residues for alanine residues (VFAEPIPANSTLVAE), eliminated this motif from the sequence at the amino acid level and did not inhibit processing of the propeptide or targeting of BL to the vacuole. Therefore, the *E. coli* endopeptidase recognition site is not utilized in processing of BL's CTPP in plants. In addition, the processing of the 35 amino acid random sequence of the BL-mutant CTPP construct 32, suggests that the propeptide may be processed in a non specific manner.

Substitution of the glutamic acid residues of CTPP with glutamine residues (mutant 36) and the basic residue lysine (mutant 37) did not disrupt CTPP processing or proper sorting of BL to the vacuole. Interestingly,

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substitution of the lysine for the acidic residues (mutant 37) did interfere with glycosylation of the CTPP suggesting that the amino acid residues flanking the N-linked glycosylation site influence its utilization in the plant secretory pathway.

A Mutational Analysis of the CTPP

The primary amino acid sequences of the CTPPs of wheat-germ agglutinin (WGA), rice lectin, and BL are not conserved; however, these CTPPs do share the potential to form amphipathic α-helices (Bednarek et al., 1990). Amphipathic α-helices are believed to function as targeting signals in mitochondrial protein import and to mediate other protein-protein interactions (Verner and Schatz, 1988). Several lines of evidence presented in this paper, however, do not support a role for the predicted amphipathic α-helical secondary structure of the CTPP in the mediation of vacuolar targeting. Mutant 33 (VFAEPIPANSTLVAE), which disrupted the predicted amphipathic α-helix by substitution of proline residues for alanine residues, was correctly processed and targeted to the vacuole. Furthermore, mutants 7 (VFAE) and 8 (VFA), in which we deleted significant portions of the propeptide needed for secondary structure, were properly sorted.

A review of the sorting data summarized in Figure 2.2 indicated that no consensus sequence for targeting was observed and that the minimum length of CTPP necessary for efficient sorting of BL to the vacuole was three amino

acids (mutant 8, VFA). These results were surprising due to the great variability tolerated in sequence length and amino acid content. Despite the lack of any apparent amino acid consensus sequence, some sequence specificity was implied by the secretion of BL mutant CTPP constructs (summarized in Figure 2.2). All of the secreted mutants demonstrated a similar pattern of secretion as the control construct 10 (total deletion of CTPP). Clearly these secreted mutants disrupted proper targeting of BL to the vacuole. However, due to the limitations in the transient expression system it is difficult to assess vacuolar content. Therefore we can not rule out the partial sorting of BL to the vacuole by some of these mutants.

A common feature associated with vacuolar proteins that have carboxyl-terminal propeptides was the presence of short stretches of hydrophobic amino acids within their sequences (Bednarek and Raikhel, 1992), which may indicate a conserved recognition mechanism. The importance of hydrophobic amino acids in the sorting signal was shown using short artificial CTPPs. These CTPPs -AAAA, AVIADA, AVIAVA, and LLVD- resulted in the retention of BL intracellularly; however, short stretches of charged amino acids lacking any hydrophobic residues (EEEE or KKKK) would lead to secretion of BL into the media. Furthermore mutants 26 (VFAEAD) and 27 (KDAEAD) demonstrated that the presence of small hydrophobic residues (alanine) within a stretch of charged amino acids resulted in the retention of BL. Overall, these results strongly suggest that hydrophobic amino acids are involved in recognition of the sorting determinant.

Comparison of the results of the deletion and glycine replacement analyses of BL-mutant CTPP construct 3 (VFAEAI), showed that the length and hydrophobicity of the CTPP were not the only characteristics to be involved in sorting. The presence of a tandem glycine positioned at the carboxy terminus of the propeptide mutant 15 (VFAEGG) and mutant 21 (VFAEAIAANSTLVAEGG) disrupted the sorting of BL and lead to secretion. In addition, the shift of the glycan close to the carboxy terminus of the CTPP disrupted the proper sorting of BL to the vacuole. Base on these results, we speculate that some component of the sorting apparatus interacts with the carboxy terminus of the propeptide.

The deletional analysis of the CTPP demonstrated that a minimum of 3 amino acids are required for proper sorting to the vacuole. A comparison of the last 3 amino acids of the extracellular BL-mutant CTPP constructs in Figure 2.2 indicated that CTPPs which have three charged amino acids (mutants 28 and 29), three glycine residues (mutants 16 to 18), or a combination of both (mutants 15 and 21) will cause secretion of BL into the media, even though there are hydrophobic amino acids present elsewhere in CTPP. Therefore, the overall amino acid content of the CTPP is not as important as the arrangement of the amino acid residues. This is similar to the requirements of the signal sequence that mediates protein translocation into the lumen of the ER (Verner and Schatz, 1988). Also, targeting to the peroxisome in plants, yeast, and mammals involves a 3-amino acid recognition sequence that is located at the carboxy terminus of the signal (Subramani,1992). In addition, targeting to the

peroxisome can be disrupted by the addition of amino acid residues to its carboxy terminus (Gould et al., 1989; Miura et al., 1992).

Mechanisms of Sorting to the Vacuole

Although amino-terminal propeptides share a common motif within their sequences, carboxyl-terminal propeptides share no common sequence identity (for reviews, see Chrispeels and Raikhel, 1992; Bednarek and Raikhel, 1992). However, both sporamin with an amino-terminal propeptide and BL with a carboxyl-terminal propeptide are targeted to the same vacuoles in leaves and roots of transgenic tobacco plants (Schroeder et al.,1993). This information in conjunction with data on vacuolar proteins that contain their sorting determinant within portions of the mature protein, such as phytohemagglutinin (Chrispeels and Raikhel, 1992) and 11S legumin (Saalbach et al., 1991), suggest that there may be multiple mechanisms or receptors for vacuolar targeting in plants.

The concept of multiple receptors or mechanisms is not unique to plants. There is evidence for a mannose-6-phosphate independent sorting of some mammalian lysosomal enzymes from the secretory pathway (Kornfeld and Mellman, 1989). Protein sorting to the yeast vacuole is mediated by multiple signals (Pryer et al., 1992). Peroxisomal targeting was also mediated by different signals located at the carboxy and amino termini (van den Bosch et al 1992; Subramani, 1992). In a deletional analysis of the propeptide that

contains a yeast vacuolar targeting signal, QRPL, Johnson et al. (1987) found that the context in which the QRPL is presented will affect the efficiency of targeting. The location of any specific determinant will be dictated by the secondary and tertiary structural requirements for any particular protein. Therefore, a critical element in the sorting process will be how the redirected protein's overall secondary structure will affect the accessibility or exposure of their targeting motif to the sorting machinery, and the possibility of multiple mechanisms or receptors would give needed flexibility to the sorting apparatus to accommodate this wide range of protein structure.

BL and WGA share 95% sequence identity at the amino acid level and therefore are presumed to share a conserved molecular structure (for review, see Raikhel and Lerner, 1991). Extensive x-ray crystallographic and sequence analyses have revealed that mature WGA is a homodimeric protein composed of 18-kD subunits. Each subunit is composed of four homologous domains, each of which consists of a tightly folded core stabilized by four disulfide bonds (Wright, 1987). Examination of the WGA crystal structure does not reveal any regions that extend from the surface. Therefore based on the crystallographic data, we predicted that the CTPP is more exposed on the surface of the lectin or may extend out from it, allowing it to interact with components of the sorting machinery.

In a broader context, we can speculate on the type of properties that a factor or protein would possess to interact with the sorting determinant. From our results, one could envision a protein or factor which possesses binding

properties similar to some chaperones or heat shock proteins that bind to a wide range of diverse sequences and show a higher affinity for binding to hydrophobic residues (Flynn et al., 1991). As to date, no sorting receptor has been isolated from yeast or plants for targeting to the vacuole. We hope that the information gained from our analysis of the CTPP will facilitate the identification of a receptor or binding factor involved in the sorting of secretory proteins to the plant cell vacuole.

Since the publication of this paper, receptors for yeast carboxypeptidase Y and plant amino-terminal propeptides have been identified. This research is discussed in detail in Chapter 1. In addition, a number of the short CTPPs (constructs 2, 3, 11 and 12) as described in Figure 2.2 were fused to the C-terminus of the secreted protein, cucumber chitinase, and were shown to direct only a portion of the chimeric proteins to the vacuole (data not shown). The inefficient sorting of these constructs is most likely due to the limited accessibility of the short CTPPs to the sorting apparatus (Bednarek and Raikhel, 1991).

MATERIALS AND METHODS

All standard recombinant DNA procedures used in this study were carried out as described in Sambrook et al. (1989), unless otherwise noted. DNA restriction and modifying enzymes were obtained from New England BioLabs (Beverly, MA). All other reagents, unless specified, were purchased from Sigma.

Preparation of BL-mutant CTPP Constructs

All barley lectin (BL) mutant carboxyl-terminal propeptide (CTPP) constructs were prepared by site-specific mutagenesis as described in Bednarek et al. (1990) with the following exceptions. BL-mutant CTPP clones were constructed either by modification of the CTPP coding region of the wild-type clone described in Wilkins et al. (1990), or by the addition of specific nucleotide sequences between the final codon for the mature protein and the stop translation codons for the *ctpp*$^-$ clone described in Bednarek et al. (1990). The region encoding the CTPP (nucleotides 607-651) of the barley lectin *wt* cDNA clone (Wilkins et al., 1990) was modified to code for the amino acids describe in Figure 2.2 using the synthetic mutagenic oligonucleotides below.

Mutant #01 5'-GCTGCGACGGTGTCAACTCCACTCTTGTCG-3'

Mutant #02 5'-GAGGCCATTGCCGCCTGATGATCTTGCTAATGGC-3'

Mutant #03 5'-CGCCGAGGCCATCTGATGATCTTGCTAATG-3'

Mutant #11 5'-GCTGCGACGGTGTCTCCACTCTTGTCGCA-3'

Mutant #12 5'-CGGCTGCGACGGTCTTGTCGCAGAATGATGA-3'

Mutant #21 5'-CACTCTTGTCGCAGAAGGAGGTTGATGATCTTGCTAATGG-3'

Mutant #33 5'-GTCTTCGCCGAGCCGATCCCGGCCAACTCCACTC-3'

Mutant #34 5'-GGCTGCGACTATGTCTTCG-3'

Mutant #35 5'-GGCGGCTGCGACTATTGGTTCGCCGAGGCCATCGCC-3'

Mutant #36 5'-GTCTTCGCCCAGGCCATC-3'& 5'-CTTGTCGCACAATGATGATC-3'

Mutant #37 5'-GTCTTCGCCAAGGCCATC-3'& 5'-CTTGTCGCAAAATGATGATC-3'

The synthetic mutagenic oligonucleotides listed below were used to insert a nucleotide sequence coding for a specific amino acid sequence (described in Figure 2.2) between the final codon of the mature protein and the stop translation codons in the *ctpp*- clone (designated Mutant #10) described in Bednarek et al., (1990).

Mutant #04 5'-CGGCTGCGACGGTGTCTTCGCAAACTCCACTTGATGATCTTGCTAATG-3'

Mutant #05 5'-CGGCTGCGACGGTGTCTTCGCAGGTTCCACTTGATGATCTTGCTAATG-3'

Mutant #06 5'-CGGCTGCGACGGTGTTTTTGCTGAAGCATGATGATCTTGCTAATG-3'

Mutant #07 5'-CGGCTGCGACGGTGTTTTTGCAGAATGATGATCTTGCTAATG-3'

Mutant #13 5'-CGGCTGCGACGGTGTTTTTGCAGAAGCTGGATGATGATCTTGCTAATG-3'

Mutant #14 5'-CGGCTGCGACGGTGTTGGTGCTGAAGCAGGATGATGATCTTGCTAATG-3'

Mutant #15 5'-CGGCTGCGACGGTGTTTTTGCAGAAGGTGGATGATGATCTTGCTAATG-3'

Mutant #16 5'-CGGCTGCGACGGTGTGTTTGCAGGAGGTGGATGATGATCTTGCTAATG-3'

Mutant #17 5'-CGGCTGCGACGGTGTTTTTGGAGGAGGAGGATGATGATCTTGCTAATG-3'

Mutant #18 5'-CGGCTGCGACGGTGTTGGAGGAGGTGGAGGATGATGATCTTGCTAATG-3'

Mutant #19 5'-CGGCTGCGACGGTGTGTTTGCGGGATGATGATCTTGCTAATG-3'

Mutant #20 5'-CGGCTGCGACGGTGTTTTTGGGTGATGATCTTGCTAATG-3'

Mutant #23 5'-CGGCTGCGACGGTGCGGTTATTGACGTCGCATGATGATCTTGCTAATG-3'

Mutant #24 5'-CGGCTGCGACGGTGCAGTTATTGCTGTCGCATGATGATCTTGCTAATG-3'

Mutant #25 5'-CGGCTGCGACGGTGCTGCTGCAGCATGATGATCTTGCTAATG-3'

Mutant #26 5'-CGGCTGCGACGGTGTTTTTGCAGAAGCAGACTGATGATCTTGCTAATG-3'

Mutant #27 5'-CGGCTGCGACGGTAAAGATGCAGAGGCAGACTGATGATCTTGCTAATG-3'

Mutant #28 5'-CGGCTGCGACGGTGAGGAGGAGGAATGATGATCTTGCTAATG-3'

Mutant #29 5'-CGGCTGCGACGGTAAGAAGAAGAAATGATGATCTTGCTAATG-3'

Mutant #30 5'-CGGCTGCGACGGTCTCCTTGTTGACTGATGATCTTGCTAATG-3'

Mutant #31 5'-CGGCTGCGACGGTCCAATTAGACCATGATGATCTTGCTAATG-3'

Uracil-containing single stranded of BL-mutant CTPP construct #3 was prepared from bacteriophage M13KO7 grown on the host *dut⁻ ung⁻ F⁺ Escherichia coli* strain CJ236 harboring the BL-mutant CTPP construct 3 cDNA in pUC118 (Vieira and Messing, 1987). The synthetic mutagenic oligonucleotides listed below were used to delete the nucleotide sequences encoding the amino acid sequences as desribed in Figure 2.2.

Mutant #08 5'-ACGGTGTCTTCGCCTGATGATCTTGCTAATG-3'

Mutant #09 5'-GCGACGGTGTCTTCTGATGATCTTGCTAAT-3'

The region encoding the CTPP (nucleotides 607-651) of the barley lectin *wt* cDNA clone (Wilkins et al., 1990) was modified to code for the amino acids describe in Figure 2.2 using the synthetic mutagenic oligonucleotides below. The barley lectin *wt* cDNA clone was first mutagenized using oligo A was isolated and verified by ^{35}S-dideoxy sequencing (Sanger et al., 1977). Uracil-containing single stranded of this cDNA clone was prepared as previously described. A second round of mutagenesis was performed using oligo B to

obtain a cDNA clone encoding the BL-mutant CTPP construct 22.

Mutant #22 oligo A 5'-GCCAACTCCACTCTTGTCAACGCAACTGAATGATGATCTTGCTAATG-3'

& oligo B 5'-GCCGCCGGCTCCACTC-3'

All carboxyl-terminal propeptide mutants of barley lectin were identified and selected by ^{35}S-dideoxy sequencing of single-stranded DNA. The BL-mutant CTPP cDNAs were excised from pUC118 with Xbal (New England BioLabs). BL-mutant CTPP constructs (Figure 2.2) were subcloned (Struhl, 1985) into the binary plant expression vector pGA643 (An et al., 1988) and mobilized into *Escherichia coli* DH5α. All BL-mutant CTPP cDNA as well as cDNA encoding the wild-type (Wilkins et al., 1990) and *ctpp*$^-$ (Bednarek et al., 1990) clones were subcloned into the transient expression vector pA35 (Höfte and Chrispeels, 1992) and transformed into the *E. coli* MV1193. Large-scale BL-mutant CTPP pA35 plasmid preparations were performed using the Maxi-Prep Kit as described by the manufacturer (Qiagen Inc., Chatsworth, CA.).

Plant Transformation and Shoot Tissue Culture

Tobacco plants (*Nicotiana tabacum* cv Wisconsin 38) were transformed with the binary vector pGA643 containing BL-mutant CTPP constructs 1 to 3, 10, and 33-36, as shown in Figure 2.2, and analyzed as described in Wilkins et al. (1990). Axenic shoot cultures of transformed tobacco were maintained and propagated by node cuttings on solid Murrashige and Skoog (MS) medium (Murashige and Skoog, 1962) without exogenous hormones.

Immunocytochemistry

Immunocytochemistry was performed on transgenic tobacco plants individually expressing BL mutants 1 to 3 essentially as described in Bednarek and Raikhel (1991). The primary antibody was rabbit anti-WGA antiserum (Raikhel et al., 1984) diluted 1 to 50, and control sections were incubated with nonimmune serum diluted similarly. Protein A-colloidal gold (EY Laboratories Inc., San Mateo, CA) was diluted 1 to 50.

Vacuole Isolation and Marker Enzyme Assays

Vacuole isolation and marker enzyme assays (Bednarek and Raikhel, 1991) were performed on transgenic plants expressing BL-mutant CTPP constructs. Affinity-purified BL from vacuole extracts and crude soluble protein extracts from protoplasts were examined by protein gel blot analysis as described in Wilkins et al. (1990).

Radiolabeling of Transformed Tobacco Leaf Protoplasts

Protoplasts were prepared and isolated as described previously (Bednarek and Raikhel, 1991), with the exception that the isolated protoplasts were diluted to a final concentration of 500,000 protoplasts per mL. Viable protoplasts were quantified, and pulse-labeling experiments of leaf protoplasts were performed (Bednarek et al., 1990).

Transient Gene Expression in Tobacco Leaf Protoplasts

The transient expression of BL-mutant CTPP constructs in tobacco leaf protoplasts via the PEG-mediated DNA uptake method (Bednarek et al., 1990) was performed for tobacco suspension cell culture protoplasts with some alterations. Protoplasts from tobacco plants (cv Wisconsin 38) were prepared and isolated as described previously (Bednarek and Raikhel, 1991), with the exception that after the wash the isolated protoplasts were resuspended in 30 mL of W5 solution (188 mM NaCl, 153 mM $CaCl_2 \cdot 2H_2O$, 5mM KCl, 5mM glucose, pH 5.7). Viable protoplasts were visualized by fluorescein diacetate staining (Widholm, 1972) and the yields quantitated using a hemocytometer counting chamber. Protoplasts were collected by centrifugation at (50g) for 10 min, washed with 30 mL of BaMg solution (0.6 M betaine, 15 mM $MgCl_2$, 3 mM 2-[*N*-morpholino]ethanesulpfonic acid [Mes]-KOH, pH 5.7), and resuspended to a final concentration of 1.7 X 10^6 viable protoplasts per mL with the BaMg solution. Prior to adding plasmid DNA, 5 X 10^5 protoplasts were aliquoted to 15-mL polypropylene tubes (300 μl 1.7 X 10^6 protoplast suspension per tube) and were subjected to a 45°C heat shock for 5 min.

After cooling to room temperature, a 30-μL mixture of 20 μg of a pA35 BL-mutant CTPP construct and 50 μg of sheared salmon sperm DNA was added to the protoplast suspension. The protoplast/plasmid DNA mixture was brought to a final concentration of 28% PEG-4000 with a solution containing 40% PEG-4000, 0.6 M betaine, 100 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.1 % Mes, pH 7.0. After

incubating at room temperature for 30 min, the protoplast/DNA/PEG mixture was slowly diluted with 12 volumes of W5 solution over a period of 15 min. The protoplasts were collected by centrifugation at 50g for 10 min at room temperature, and the protoplast pellet was washed with 5 mL of MS medium supplemented with 0.1 mg/L naphthaleneacetic acid, 1.0 mg/L benzyladenine, and 0.6 M betaine monohydrate (MS 0.1/1.0, 0.6 M betaine), recentrifuged, and resuspended in 1 mL of MS 0.1/1.0, 0.6 M betaine, to a final density of 5.0 X 10^5 protoplasts per mL and transferred to 12 well tissue culture plates (Costar, Cambridge, MA.).

To examine expression of the barley lectin constructs, the transiently transformed leaf protoplasts were incubated for 8 hr (pulse-chase analysis) or 20 hr (pulse-labeling) in the presence of 100 μCi Expre^{35}S^{35}S sulfur-35 protein labeling mixture (New England Nuclear Research Products), *E. coli* hydrolysate containing a mixture of 77% L-^{35}S-methionine and 18% L-^{35}S-cysteine in 50 mM tricine, 10 mM βME buffer (specific activity 1000-1100 Ci/mmol; ^{35}S-Met/Cys). If a pulse-chase analysis was performed after 8 hr of labeling, 100 μL of chase mix (100mM methionine and 50 mM cysteine [free base] in MS 0.1/1.0, 0.6 M betaine) was added and incubated for an additional 12 hr. After labeling, the protoplasts were separated from the culture medium by centrifugation at 50g for 10 min at room temperature. The protoplast pellet was resuspended in 400 μL of extraction buffer, 50 mM Tris-acetate, pH 5.0, 100 mM NaCl, and 0.6% Triton X-100. The lysate was cleared of insoluble debris by centrifugation at 16,000g for 5 min at 4°C, frozen in liquid N_2, and

stored at -70°C. The culture medium (1 mL) was filtered to remove any remaining protoplasts (Wilkins et al., 1990), and 25 μL of a 50 mg/mL BSA solution was added as a carrier protein.

Proteins in the culture media were precipitated with ammonium sulfate at 70% saturation at 4°C for 2 hr then collected by centrifugation at 10,000 rpm for 10 min at 4°C. The culture medium protein pellet was resuspended in 400 μL extraction buffer and stored at -70°C. All protein samples were thawed at room temperature and passed four times over immobilized *N*-acetylglucosamine (Pierce Chemical Co.) micro affinity columns (Mansfield et al., 1988). After extensive washing of the column with TA buffer (50 mM Tris-acetate, pH 5.0 , and 100mM NaCl), BL was eluted with 150 μL of 200 mM N-acetylglucosamine and lyophilized. The inhibition of glycoslyation by tunicamycin was performed as described in Bednarek and Raikhel, (1991). The radiolabeled barley lectin was analyzed by SDS-PAGE through 12.5% or 15% polyacrylamide gels and visualized by fluorography as detailed in Mansfield et al. (1988).

Acknowledgments

We would like to thank Olga Borkhsenious for helping with electron microscopy immunocytochemistry, Drs. Herman Höfte and Maarten Chrispeels for generously providing us with the plasmid for the transient expression vector pA35. We would also like to thank all members of our laboratory for many helpful discussions and for critical reading of this manuscript. This research was supported by grants from the National Science Foundation, Washington, DC (Grant No. DCB-9002652) and the United States Department of Energy, Washington, DC (Grant No.DE-AC02-76ERO-1338) to N.V.R.

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CHAPTER 3

ISOLATION OF A cDNA ENCODING A NOVEL GTP BINDING PROTEIN OF *ARABIDOPSIS THALIANA*

Reference: Dombrowski JE, Raikhel NV (1995). Plant Mol Biol in press.

ABSTRACT

A cDNA encoding for a 68 kDa GTP binding protein was isolated from *Arabidopsis thaliana* (*aG68*). This clone is a member of a gene family that codes for a class of large GTP binding proteins. This includes the mammalian dynamin, yeast Vps1p and the vertebrate Mx proteins. The predicted amino acid sequence was found to have high sequence conservation in the N-terminal GTP-binding domain sharing 54% identity to yeast Vps1p, 56% amino acid identity to rat dynamin and 38% identity to the murine Mx1 protein. The Northern analysis shows expression in root, leaf, stem and flower tissues, but in mature leaves at lower levels. Southern analysis indicates that it may be a member of a small gene family or the gene may contain an intron.

DISCUSSION AND RESULTS

In eukaryotic cells, GTP-binding proteins function in a wide variety of cellular processes, including signal transduction, intracellular transport of proteins, cytoskeletal organization and protein synthesis. During the last few years a unique class of high molecular weight GTPases have been identified, which have a conserved N-terminal tripartite GTP-binding domain. The members of this gene family have been shown to be involved in important cellular functions. The mammalian protein dynamin and its *Drosophila* homologue, the product of the *shibire gene* has been demonstrated to be involved in endocytosis, synaptic transmission, and neurogenisis (Obar et al., 1990; Chen et al., 1991; van der Bliek and Meyerowitz, 1991; Gout et al., 1993; Herskovits et al., 1993a,b; Robinson et al., 1993; van der Bliek et al., 1993; Damke et al., 1994; Scaife et al., 1994). In yeast two homologues have been isolated, the MGM1 protein which plays a role in mitochondrial DNA maintenance (Jones et al., 1992), and the Vps1p protein which is necessary for the proper sorting of soluble vacuolar proteins (Rothman et al., 1990; Vater et al., 1992) and for membrane protein retention in the late Golgi compartment (Wilsbach and Payne, 1993). In addition, it was recently shown that in *vps1* mutant cells Golgi as well as vacuolar membrane proteins reach the vacuole via the plasma membrane (Nothwehr et al., 1995). Another member of this family is the vertebrate Mx proteins that confers viral resistance (Staeheli et al., 1986; Horisberger et al., 1990, 1991; Pavlovic et al., 1993). We report here the first

member of this GTPase gene family to be identified in plants.

The research focus of our laboratory is to study the mechanisms of vacuolar protein sorting in plants. The MSU-DOE Plant Research Laboratories Arabidopsis Genome Sequencing Project (Newman et al., 1994) identified a partial clone whose sequence had homology to the yeast Vps1p. Due to our ongoing interest in proteins involved in the sorting of vacuolar proteins we have characterized this clone further. Using this partial clone as a probe the full length cDNA clone was isolated by plaque hybridization from an *Arabadopsis* cDNA lambda-ZAP II library made from leaf tissue ecotype *Columbia*. The lambda-ZAP II clone was converted to a pBluescript phagemid (Stratagene). The nucleotide sequence of both strands of the cDNA clone designated *Arabidopsis thaliana aG68*, was determined using the dideoxy chain termination method (Sanger et al., 1977).

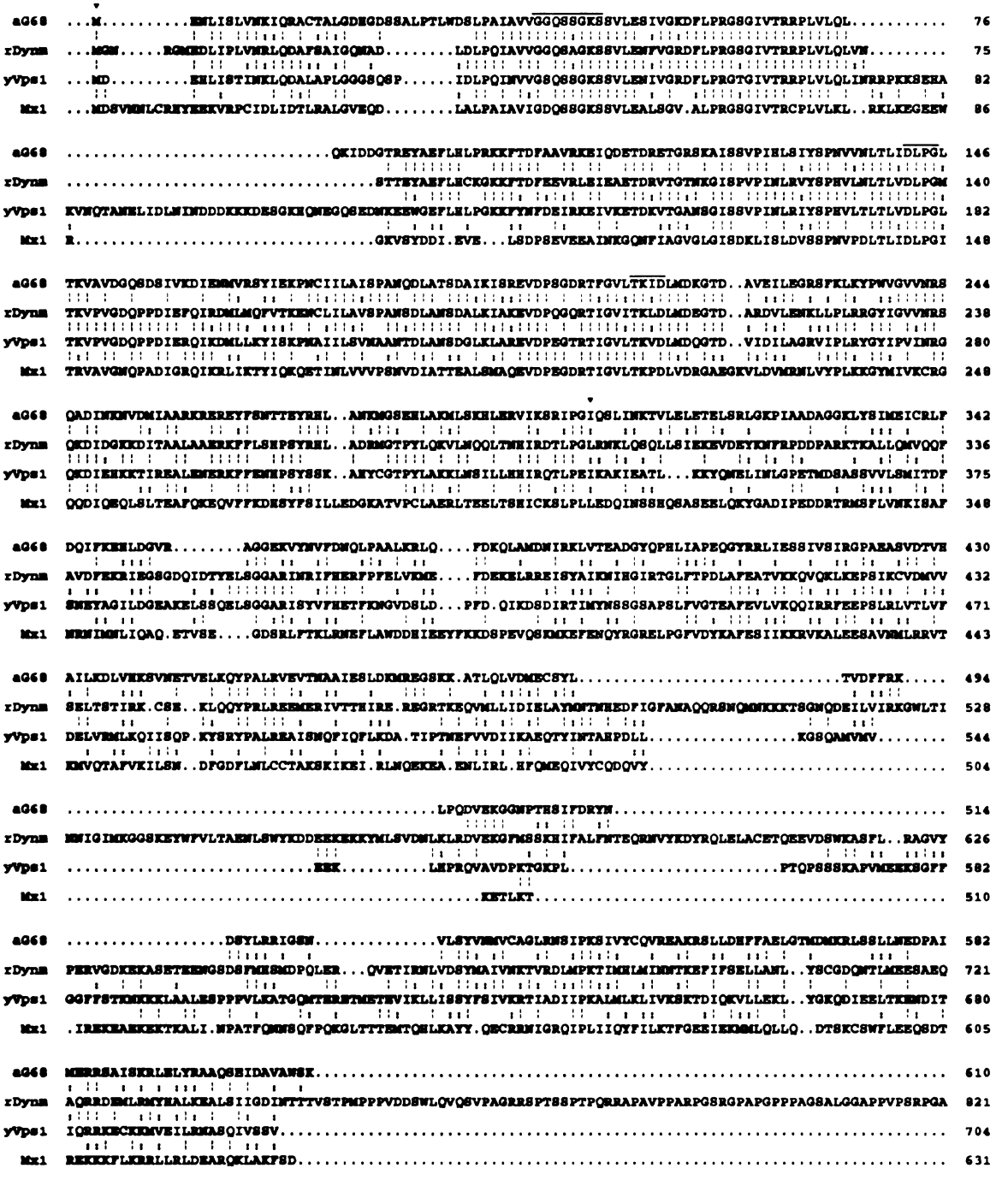
The predicted amino acid sequence of the *aG68* cDNA clone (Figure 3.1) shows no significant hydrophobic stretches, and does not appear to have a signal sequence nor a membrane spanning domain. In addition the sequence does not contain a myristylation motif at the N-terminus or a cysteine motif for prenylation at its C-terminus.

The predicted amino acid sequence of the aG68 protein was aligned (Figure 3.2) with the amino acid sequences of selected representatives of this GTPase family, yeast Vps1p (Rothman et al., 1990), rat dynamin (Obar et al., 1990), and murine Mx1 (Staeheli et al., 1986) proteins. The deduced amino acid sequence of the protein encoded by *aG68* shows a very high sequence

Figure 3.1 Sequences of the nucleotides and deduced amino acid residues of the cDNA clone *aG68*. EMBL, Genbank and DDBJ nucleotide sequence databases: accession number L38614.

```
   1  CGGCACGAGCTTCATCGACTCAAATTCAAAAACTCATCTCTCTCTATCTCTATTTCTCTGGTTCCATAGCTCACCGTCGC
  81  ATCGCAGATCTACTCCTTCCGCAATAAATTTTACCGGCGGAGGTATCAGATCTCGCCGATCTGTTGTAGCAGCTACTGTA

   1                                                          M  E  N  L  I  S  L  V  N  K  I
 161  TTTTGGGCTTCTCATTTGATATTGGGGAAACGAGGAGTAGAGGACGATGGAAAATCTGATCTCTCTGGTTAACAAGATAC

  11  Q  R  A  C  T  A  L  G  D  H  G  D  S  S  A  L  P  T  L  W  D  S  L  P  A  I  A
 241  AGAGAGCTTGCACGGCTTTAGGAGACCATGGAGACTCCAGCGCTTTACCTACTCTTTGGGATTCCTTGCCTGCGATCGCC

  39  V  V  G  G  Q  S  S  G  K  S  S  V  L  E  S  I  V  G  K  D  F  L  P  R  G  S  G
 321  GTCGTTGGTGGTCAGAGCTCAGGGAAGTCTTCAGTCCTGGAGAGCATCGTGGGAAAGGACTTTTTACCCCGTGGATCTGG

  66  I  V  T  R  R  P  L  V  L  Q  L  Q  K  I  D  D  G  T  R  E  Y  A  E  F  L  H
 401  CATTGTTACTCGAAGGCCCCTTGTCTTACAGTTGCAAAAGATCGATGATGGAACCCGGGAGTATGCAGAGTTTCTTCACC

  92  L  P  R  K  K  F  T  D  F  A  A  V  R  K  E  I  Q  D  E  T  D  R  E  T  G  R  S
 481  TCCCGAGGAAAAAGTTTACTGATTTTGCTGCTGTGAGGAAGGAGATTCAAGATGAGACTGACAGAGAGACTGGACGCAGC

 119  K  A  I  S  S  V  P  I  H  L  S  I  Y  S  P  N  V  V  N  L  T  L  I  D  L  P  G
 561  AAGGCTATTTCTAGTGTTCCCATTCACCTTAGCATATACTCTCCCAATGTTGTCAACTTGACACTGATAGATCTTCCAGG

 146  L  T  K  V  A  V  D  G  Q  S  D  S  I  V  K  D  I  E  N  M  V  R  S  Y  I  E
 641  GCTTACAAAAGTTGCTGTTGATGGACAATCTGATAGTATAGTGAAGGACATTGAAAACATGGTTCGGTCCTACATTGAAA

 172  K  P  N  C  I  I  L  A  I  S  P  A  N  Q  D  L  A  T  S  D  A  I  K  I  S  R  E
 721  AGCCCAACTGCATCATTTTGGCAATCTCACCTGCAAACCAAGATCTTGCTACCTCAGATGCAATTAAAATTTCCCGTGAG

 199  V  D  P  S  G  D  R  T  F  G  V  L  T  K  I  D  L  M  D  K  G  T  D  A  V  E  I
 801  GTTGATCCATCGGGGGACAGAACATTTGGTGTCTTGACAAAGATTGATCTTATGGACAAGGGGACGGATGCAGTGGAAAT

 226  L  E  G  R  S  F  K  L  K  Y  P  W  V  G  V  V  N  R  S  Q  A  D  I  N  K  N
 881  TCTGGAAGGGAGATCTTTTAAACTTAAATATCCGTGGGTTGGTGTCGTCAACCGTTCCCAAGCAGATATTAACAAGAATG

 252  V  D  M  I  A  A  R  K  R  E  R  E  Y  F  S  N  T  T  E  Y  R  H  L  A  N  K  M
 961  TCGACATGATTGCGGCTCGGAAAAGAGAGAGGGAGTACTTTTCCAATACTACTGAGTATAGGCACCTTGCTAATAAAATG

 279  G  S  E  H  L  A  K  M  L  S  K  H  L  E  R  V  I  K  S  R  I  P  G  I  Q  S  L
1041  GGTTCCGAGCATTTGGCAAAGATGCTCTCCAAGCATCTAGAACGTGTGATCAAGTCGAGAATTCCTGGCATTCAGTCACT

 306  I  N  K  T  V  L  E  L  E  T  E  L  S  R  L  G  K  P  I  A  A  D  A  G  G  K
1121  TATTAACAAAACAGTATTAGAGCTGGAAACTGAACTAAGTCGCCTTGGAAAGCCTATTGCAGCTGATGCAGGGGGGAAGT

 332  L  Y  S  I  M  E  I  C  R  L  F  D  Q  I  F  K  E  H  L  D  G  V  R  A  G  G  E
1201  TGTACTCAATAATGGAGATATGTCGGCTTTTTGATCAAATATTCAAAGAGCATCTTGATGGAGTGCGTGCTGGTGGTGAA

 359  K  V  Y  N  V  F  D  N  Q  L  P  A  A  L  K  R  L  Q  F  D  K  Q  L  A  M  D  N
1281  AAAGTGTACAACGTGTTTGATAACCAGCTTCCTGCGGCTCTGAAGAGACTCCAATTTGACAAGCAGCTAGCGATGGACAA

 386  I  R  K  L  V  T  E  A  D  G  Y  Q  P  H  L  I  A  P  E  Q  G  Y  R  R  L  I
1361  CATCCGGAAGCTGGTCACTGAGGCTGATGGTTACCAGCCTCACTTGATTGCTCCTGAGCAAGGTTACCGTCGTCTCATTG

 412  E  S  S  I  V  S  I  R  G  P  A  E  A  S  V  D  T  V  H  A  I  L  K  D  L  V  H
1441  AGTCTTCTATTGTCTCCATCAGAGGCCCTGCTGAAGCATCTGTTGACACCGTTCATGCTATCTTAAAGGATCTGGTTCAC

 439  K  S  V  N  E  T  V  E  L  K  Q  Y  P  A  L  R  V  E  V  T  N  A  A  I  E  S  L
1521  AAGTCTGTGAATGAAACTGTGGAACTAAAACAATACCCAGCTCTGAGAGTGGAGGTGACAAATGCGGCGATAGAGTCGCT

 466  D  K  M  R  E  G  S  K  K  A  T  L  Q  L  V  D  M  E  C  S  Y  L  T  V  D  F
1601  GGATAAAATGCGGGAAGGAAGTAAGAAAGCAACACTGCAGCTGGTTGACATGGAGTGCAGTTACCTCACTGTTGATTTCT

 492  F  R  K  L  P  Q  D  V  E  K  G  G  N  P  T  H  S  I  F  D  R  Y  N  D  S  Y  L
1681  TCAGGAAACTTCCCCAGGATGTTGAGAAGGGTGGTAACCCCACACACTCCATTTTCGACCGCTACAACGATTCCTATCTC

 519  R  R  I  G  S  N  V  L  S  Y  V  N  M  V  C  A  G  L  R  N  S  I  P  K  S  I  V
1761  AGACGAATCGGATCCAATGTTTTGTCTTACGTGAACATGGTCTGTGCTGGCCTGCGGAATTCAATCCCCAAGTCCATCGT

 546  Y  C  Q  V  R  E  A  K  R  S  L  L  D  H  F  F  A  E  L  G  T  M  D  M  K  R
1841  ATACTGCCAAGTCCGAGAAGCGAAGCGCAGTCTCCTCGACCATTTCTTTGCGGAGCTCGGTACCATGGATATGAAGAGGC

 572  L  S  S  L  L  N  E  D  P  A  I  M  E  R  R  S  A  I  S  K  R  L  E  L  Y  R  A
1921  TCTCGTCGCTATTGAACGAAGATCCAGCAATCATGGAGAGACGCAGTGCCATCTCAAAGCGGCTAGAATTGTATCGAGCA

 599  A  Q  S  E  I  D  A  V  A  W  S  K  *
2001  GCCCAATCCGAGATCGATGCTGTTGCTTGGTCCAAGTGATACCGGCATGTCATGTCCACTGTTTTGCTCGGTTCTGGTCG

2081  GTGTGGCTCAGACTCGGAGCAGAGATTTAGGGTCTGTAATTTGTATAAGATGATCTTCCCGATACCATGCAGTATCGTTT
2161  TATATAACATCCACATTGTTTGTCCTACCTCTATGTTTTTTGTCCATCACCCGATATGTTACGTATCGTTTTATAAAAAA
2241  AAAAAAAAAAAAA
```

Figure 3.2 Alignment of the predicted amino acid sequences of *aG68*, rat dynamin (rDynm), yeast Vps1p, and murine Mx1 proteins. The sequences were aligned using the University of Wisconsin (Madison), Genetics Computer Group Sequence Analysis Software Package (version 7.0) were carried out on a version 7.3 UNIX computer. The selected sequences were obtained from published sequences (Obar et al., 1990; Rothman et al., 1990; Staeheli et al., 1986). Arrows mark the boundaries containing the region of greatest similarity. This region was selected visually. Vertical dashes, identity and colons conservative substitutions. The guanine-nucleotide consensus elements are overlined (Obar et al., 1990; Rothman et al., 1990).


aG68
rDynm
yVps1
Mx1

conservation in the N-terminal (≈300 amino acids) GTP binding domain. The amino acid sequence of *aG68* shows 65% sequence similarity to yeast Vps1p (54% identity), 68% sequence similarity to rat dynamin (56% identity), and 57% sequence similarity to murine Mx1 (38% identity) within the GTP binding domain. It has been previously reported that within this N-terminal domain rat dynamin showed 66% sequence identity to yeast Vps1p and 43% identity with murine Mx1, and Mx1 shares 44% sequence identity the Vps1p from yeast (Obar et al., 1990). However all the sequences were found to diverge beyond the GTP binding domain suggesting that they may perform different cellular functions. It has been speculated that the C-terminal domains of these proteins may be involved in protein-protein interactions and thereby determining their mode of action.

The dynamin subfamily of homologous proteins all possess a basic, proline rich C-terminal region which contains binding sites for microtubules and SH3 domains (Obar et al., 1990; Chen et al., 1991; van der Bliek and Meyerowitz, 1991; Gout et al., 1993; Herskovits et al., 1993a,b; Robinson et al., 1993; van der Bliek et al., 1993; Damke et al., 1994; Scaife et al., 1994). A sequence comparison of rat dynamin and its human homologue shows that they share 99% amino acid identity (van der Bliek et al., 1993). However the *Drosophila* gene shibire is the dynamin homologue with 68% sequence identity (81% similarity- conserved amino acid substitutions) with rat dynamin, and also contains a C-terminal extension of comparable length and composition (Chen et al., 1991; van der Bliek and Meyerowitz, 1991). Unlike dynamin, the

predicted sequence aG68p, the Vps1p, and Mx1 proteins lack the basic proline rich C-terminal region.

The results for Northern blot analysis were obtained using either total isolated RNA or poly(A^+) mRNA from various tissues. We found that in *Arabidopsis* the *aG68* gene is expressed at different levels in root, leaf, stem and flower tissues (Figure 3.3). Surprising is the lower level of expression exhibited in mature leaf tissue. However, in situ hybridization using full length *aG68* antisense RNA probes, showed that *aG68* transcript levels to be distributed evenly throughout the leaf tissue (Figure 3.4). It should be noted that, this lower level of expression in mature leaf tissue has been shown for other genes whose products are associated with the secretory pathway (Bar-Peled et al., 1995).

To investigate the number of related genes in *Arabidopsis*, Southern blot analysis was performed (Figure 3.5). Total genomic DNA was isolated from *Arabidopsis thaliana* ecotypes Columbia and RLD, digested with restriction enzymes and hybridized under stringent conditions with a probe generated by random primer labeling of the full length clone minus the poly-A-tail. It should be noted that the banding patterns from both ecotypes used were identical. The presence of two bands in the Hind III digestion and the four bands in the Eco RI digestions suggest a small gene family or an intron in the genomic sequence. If *aG68* is a member of a small gene family in *Arabidopsis* then there may be different expression patterns of these genes in various tissues and in response to different environmental conditions. This can be addressed by probing

Figure 3.3 Northern blot analysis for *aG68* expression in plant tissues. Northern blot analysis for the expression of *aG68* of 30μg total RNA isolated from different plant tissues, root, leaf, stem and flower of *Arabidopsis thaliana*. Equal loading of RNA in each lane was verified by ethidium bromide staining of the agarose gel and by a control northern using *aARF* gene (data not shown) which is expressed at similar levels in all tissues tested (Bar-Peled et al., 1995). Hybridization with aG68 cDNA labelled by random priming with ^{32}P was performed for 18 h at 42°C in 5X SSC, 5X Denhardt's solution 50% formamide, 0.01% SDS, 100μg/ml salmon sperm DNA, 50mM sodium phosphate pH 6.5. Final washing was in 0.2X SSC, 0.01% SDS at 60 °C.

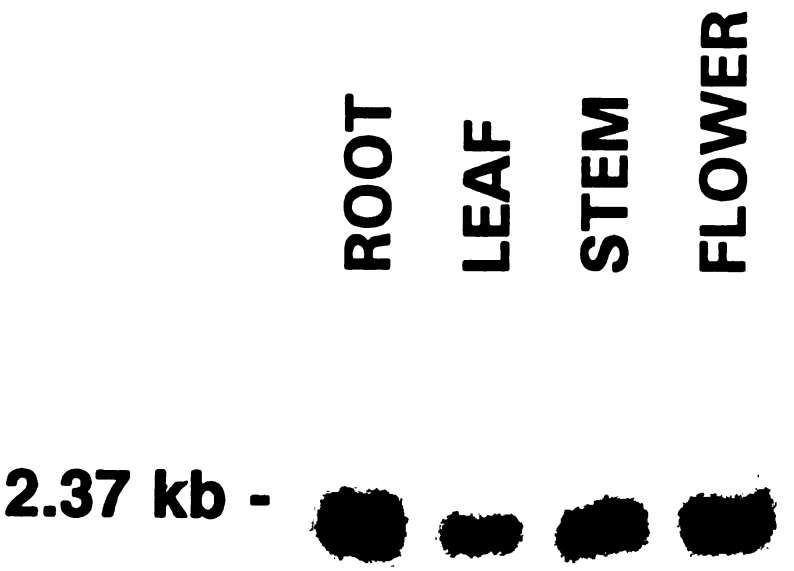

ROOT
LEAF
STEM
FLOWER
2.37 kb -

Figure 3.4 In situ localization of *aG68* transcript in wild type *Arabidopsis* **leaves.**

(A). Control transverse section of *Arabidopsis* (ecotype RLD) leaf hybridized with dioxigenin-labeled sense RNA of *aG68*.

(B). Transverse section of *Arabidopsis* (ecotype RLD) leaf hybridized with dioxigenin-labeled antisense RNA of *aG68*.

In situ hybridizations were done as described in Bar-Peled et al., (1995).

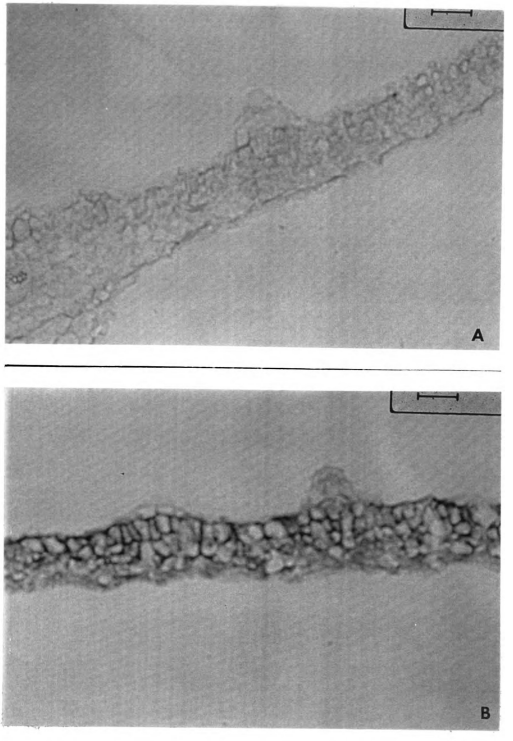
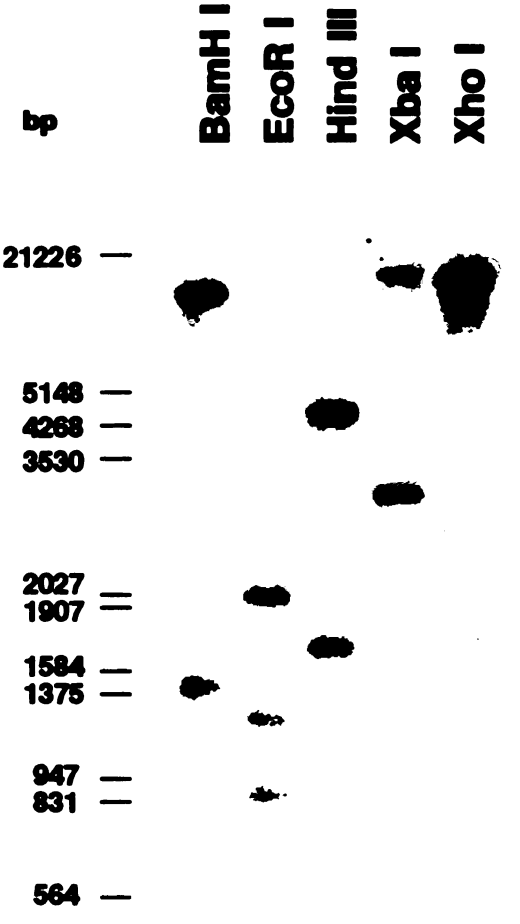

A
B

Figure 3.5 Southern blot analysis of the *aG68* gene.

Each lane was loaded with 5μg of genomic DNA digested with the restriction enzyme as indicated. The DNA was separated on 0.75% agarose gel and transferred to Hybond-N nylon membrane. The blot was then probed with *aG68* cDNA without its poly A tail, labelled by random priming with ^{32}P was performed for 18 h at 60°C in 6X SSC, 5X Denhardt's solution, 0.2% SDS, and 100μg/ml salmon sperm DNA. Final washing was in 0.2X SSC, 0.01% SDS at 60 °C.


bp
BamH I
EcoR I
Hind III
Xba I
Xho I
21226
5148
4268
3530
2027
1907
1584
1375
947
831
564

Northern blots with a 3′ non conserved region of the cDNA.

Although the predicted amino acid sequence of *aG68* has 65% sequence similarity (54% identity) in its N-terminal domain and has 53% overall sequence similarity (36% identity) to the yeast Vps1p, we were unable to complement the *vps1* yeast mutant (Vater et al., 1992). The reason may be that some plant proteins are unable to integrate into the yeast sorting apparatus, because their sequence diverges enough to disrupt the specificity of protein-protein interactions (Welters et al., 1994). However there are examples where plant genes can complement yeast secretory mutants for example, the *aERD2* gene (Lee et al., 1993), the receptor involved in retention of soluble proteins in the endoplasmic reticulum and the *aRAB6* gene (Bednarek et al., 1994), a small GTP binding protein. We will attempt to create a mutant phenotype in *Arabidopsis* by suppressing the expression of the *aG68* gene by antisense and by expressing a construct of a dominant mutation (Vater et al., 1992) of this gene in order to ascertain its function (see Figure 3.6 for constructs to be used). In summary we have identified and cloned from *Arabidopsis thaliana* the first plant member of a unique class of GTPases.

Figure 3.6 Schematic Representation of the Double Transgene Expression Cassettes of *aG68* and wild type Barley Lectin in the Binary Vector pMOG800.

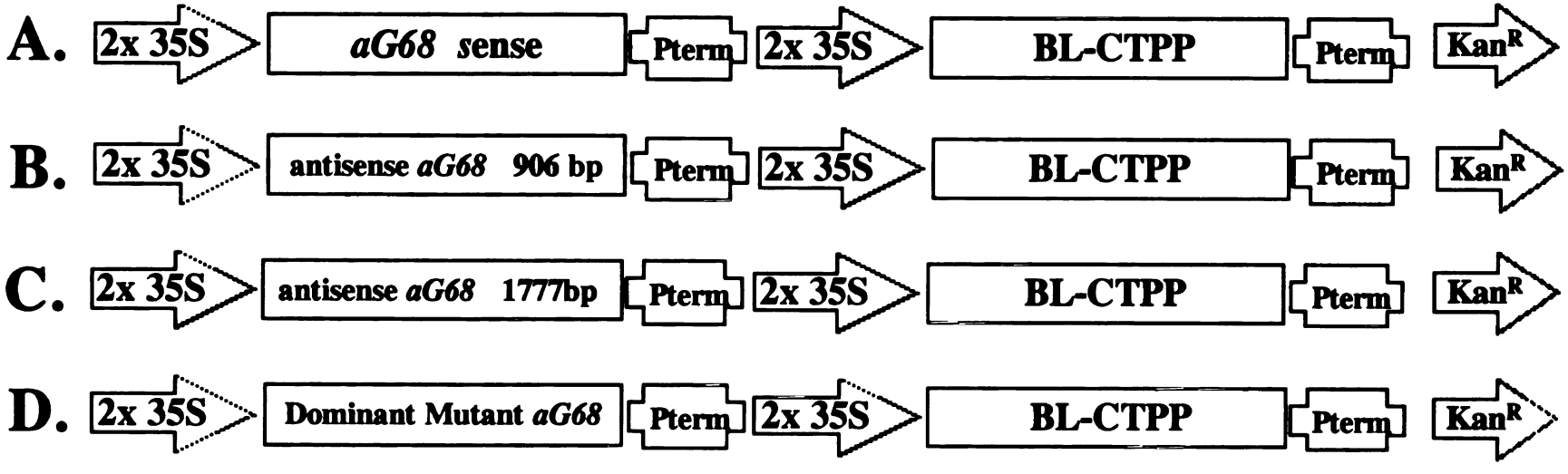
Description of the pBS843 XKEH expression cassette and the binary vector pMOG800 can be found in Chapter 4 in the material and methods section. Antibiotic resistance: nopaline synthase promoter (nos):: neomycin phosphotransferase II (NPTII):: nos terminator (Angenon et al., 1994).

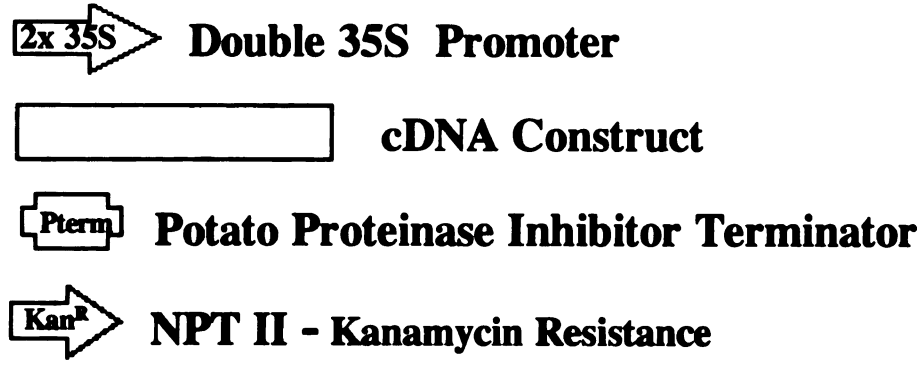
(A). Contains CDNA constructs; the full length *aG68* cDNA clone in a sense orientation and the wild type barley lectin clone containing its carboxy-terminal propeptide (BL + CTPP) as the reporter protein for vacuolar sorting (for information concerning this protein see Chapter 2).

(B). Contains CDNA constructs; a 906 bp DraI-PstI fragment (nucleotides 1-906) of the *aG68* cDNA clone in an antisense orientation and the wild type BL + CTPP clone.

(C). Contains CDNA constructs; a 1777 bp BamHI fragment (nucleotides 1-1777) of the *aG68* cDNA clone in an antisense orientation and the wild type BL + CTPP clone.

(D). Contains CDNA constructs; a dominant mutation construct in which the coding region for the GTP-binding domain has been deleted (deleted nucleotides 238-906, using HincII and DraI restiction sites) from the *aG68* cDNA clone and the wild type BL + CTPP clone.


A.
2x 35S
aG68 sense
Pterm
2x 35S
BL-CTPP
Pterm
KanR
B.
2x 35S
antisense aG68 906 bp
Pterm
2x 35S
BL-CTPP
Pterm
KanR
C.
2x 35S
antisense aG68 1777bp
Pterm
2x 35S
BL-CTPP
Pterm
KanR
D.
2x 35S
Dominant Mutant aG68
Pterm
2x 35S
BL-CTPP
Pterm
KanR


2x 35S
Double 35S Promoter
cDNA Construct
Pterm
Potato Proteinase Inhibitor Terminator
KanR
NPT II - Kanamycin Resistance

Acknowledgements

We would like to thank Dr. Scott Uknes and Dr. Eric R. Ward of CIBA-GEIGY for their generous gift of the *Arabidopsis thaliana* leaf lambda ZAPII cDNA library. Dr. Tom Stevens for providing us the yeast *vps1* mutant stain and control plasmid. I would also like to give special thanks to Amy Sandul for the isolation of the genomic DNA used in these analysis, and Dr. Alex S. Conceicao for performing the in situ hybridizations. This research was supported by grants from the National Science Foundation, Washington, DC (Grant # DCB-9002652) and the United States Department of Energy, Washington, DC (Grant # DE-F02-90ER-20021) to N.V.R.

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CHAPTER 4

THE DEVELOPMENT OF A GENETIC SCREEN FOR PLANT VACUOLAR PROTEIN SORTING MUTANTS.

INTRODUCTION

Currently, little is known about the mechanisms or the machinery involved in targeting proteins to the plant cell vacuole. Many proteins are delivered to the vacuole by way of the secretory pathway. The sorting of proteins to the vacuole is mediated by targeting signals contained in either an amino-terminal propeptide (NTPP), a carboxyl-terminal propeptide (CTPP), or a mature portion of the protein (for reviews see, Chrispeels and Raikhel, 1992; Bednarek and Raikhel, 1992; Vitale and Chrispeels, 1992; Nakamura and Matsuoka, 1993). Although proteins utilizing either NTPP or CTPP targeting signals are delivered to the same vacuoles (Schroeder et al., 1993) and the targeting signals of barley lectin (CTPP) and sporamin (NTPP) have been shown to be functionally interchangeable (Matsuoka et al., 1995), there is now strong evidence for multiple receptors as well as mechanisms for the targeting of soluble proteins to the vacuole.

While CTPPs have no consensus sequence (Dombrowski et al., 1993; Neuhaus et al., 1994), NTPPs display a common motif (for reviews see, Chrispeels and Raikhel, 1992; Nakamura and Matsuoka, 1993) which allowed for the isolation of a putative vacuolar NTPP receptor (Kirsh et al, 1994). Binding assays with the isolated receptor protein demonstrated that NTPP peptides were able to compete for binding, while a mutant NTPP peptide and the CTPP of barley lectin (BL) could not. The inability of CTPP targeting signals to compete for binding indicates that there are multiple receptors involved in the

targeting process. This fact, coupled with the observation that when very high levels of a vacuolar chitinase (CTPP mediated targeting) were produced transiently, secretion of chitinase was observed (Neuhaus et al., 1994), indicating saturation of the sorting apparatus, suggests the presence of a CTPP receptor. In addition, the existence of a CTPP receptor is supported by the fact that the CTPPs from both BL and tobacco chitinase were able to redirect a normally secreted protein to the vacuole (Bednarek and Raikhel, 1991; Neuhaus et al., 1991). Results obtained from a detailed mutational analysis of BL's CTPP demonstrated that a minimum of three exposed amino acids was sufficient to direct BL to the vacuole, and the interaction of the sorting apparatus with the CTPP occurs at the carboxy-terminus of the propeptide (Dombrowski et al., 1993). These observations suggest that the CTPP is most likely recognized by a low selectivity binding site(s), the saturation properties and specificity of which make it extremely difficult to isolate by biochemical means.

In yeast the gene *VPS34* codes for a phosphotidylinositol 3-kinase (PI 3-kinase) which has been shown to be involved in the targeting of proteins to the vacuole (Shu et al., 1993; Stack et al., 1993). Recently a specific inhibitor of mammalian PI 3-kinase wortmannin (Yano et al., 1993; Thelen et al., 1994; Woscholski et al., 1994) was used in plants to investigate its effects on the delivery of NTPP and CTPP containing proteins to the vacuole (Matsuoka et al., 1995). Pulse chase analyses in tobacco BY-2 cells indicate that wortmannin at lower concentrations caused secretion of proteins utilizing CTPP targeting signals, while NTPP mediated transport to the vacuole displayed almost no

sensitivity at this concentration of inhibitor. This differential sensitivity to wortmannin suggests two different mechanisms for sorting of plant soluble vacuolar proteins.

In yeast the identification and utilization of *vps* (vacuolar protein sorting) mutants as well as the *sec* mutants which affect protein movement along the secretory pathway have played a vital role in the investigation of the mechanisms of protein transport (reviews, Klionsky et al., 1990; Raymond et al., 1992 a,b; Pryer et al., 1992). A number of genetic selections have been used to isolate vacuolar protein sorting mutants in yeast. The first screen to identify yeast vacuolar protein sorting mutants was originally designed to isolate mutants in proteinase activity. In yeast, vacuolar proteinases need to be processed in order to be activated, and this activation is believed to occur in the vacuole. This screen identified 16 complementation groups, which were designated the *pep* mutants (Jones, 1977). A different approach was used by Stevens group; they screened for the mislocalization of CPY to the cell surface and isolated 19 complementation groups (Rothman and Stevens 1986; Rothman et al., 1989). A third screen utilized the fact that yeast require cell surface invertase (*SUC2*) activity to ferment sucrose. Using *suc*2 mutant yeast cells it was possible to isolate suc + isolates which mislocalize the normally vacuolar localized CPY-invertase fusion protein, to the cell surface (Banakaitis, et al., 1986; Robinson et al., 1988). This screen identified 33 complementation groups, which were classified according to vacuolar morphology. The two later screens had 12 complementation groups in common, they were combined and

designated as *vps* (vacuolar protein sorting) mutants (Rothman et al., 1989). Most mutants appear only to perturb sorting of soluble vacuolar proteins and not vacuolar membrane associated proteins. In addition, the isolation and propagation of *vps* null mutants have shown that many of these genes are not essential for cell viability. Furthermore, *vps* mutants display a variety of vacuolar morphologies (Raymond et al., 1992a). Currently there are 46 *vps* mutants which affect the delivery of proteins to the yeast vacuole (Klionsky et al., 1990; Raymond et al., 1992b). This genetic approach has played a major role in the identifying components and addressing the questions concerning the mechanisms of vacuolar protein sorting in yeast. Recently, the *VPS10* gene was identified as the specific receptor for carboxypeptidase Y (Marcusson et al., 1994).

In contrast to yeast, only a small number of potential components of the plant vacuolar sorting apparatus have been identified; the *Arabidopsis* syntaxin homologue *aPEP12* (Bassham et al., 1995), two small GTPases associated with transport vesicles in pumpkin (Shimada et al., 1994), a large GTP-binding protein from *Arabidopsis (aG68)* (Dombrowski and Raikhel, 1995), an *Arabidopsis* phosphotidylinositol 3-kinase (PI 3-kinase) homologue *AtVPS34* (Welters et al., 1994), and the putative receptor for NTPP mediated targeting from pea (Kirsh et al., 1994). The lack of plant protein sorting mutants or a cell free assay/transport system has hindered further progress in the identification of components of the sorting machinery.

A wide range of biochemical approaches have been used in an attempt

to characterize a CTPP receptor. Binding assays and cross-linking studies using radiolabeled synthetic CTPP or *E.coli* expressed proBL (BL + CTPP) and isolated Golgi membranes from tobacco or an *E. coli* expression library were not successful (M. Schroeder, T. Reynolds, J.E. Dombrowski, and N.V. Raikhel unpublished results). In addition, a similar approach as was used for the isolation of a putative NTPP-receptor (Kirsch et al., 1994) was tried using CTPP. However, specific binding of the CTPP to proteins obtained from clathrin-coated vesicles of developing pea cotyledons was not observed (T. Kirsh, J.E. Dombrowski, L. Beevers and N.V. Raikhel unpublished results). Therefore, to compliment molecular and biochemical approaches, genetic studies were initiated using *Arabidopsis thaliana*, an excellent model plant for molecular genetics (Estelle and Somerville, 1986; Somerville, 1989; Koncz and Rédei, 1994; Koornneef, 1994; Meyerowitz, 1994; Scholl et al., 1994). In this chapter it has been shown that when BL + CTPP is transformed into *Arabidopsis*, it is correctly processed and targeted to the vacuoles in roots and leaves. Therefore the creation of vacuolar protein sorting mutants in *Arabidopsis* will provide an excellent opportunity to isolate the CTPP receptor. To create plant vacuolar sorting mutants, *Arabidopsis thaliana* plants transformed with BL were treated with ethylmethane sulfonate (EMS) (Koornneef et al., 1982; Malmberg 1993; Feldmann et al., 1994). The intracellular wash fluids from M_2 plants were screened for secretion of BL. The preliminary screen identified three putative mutants. The information gathered from this study has led to the development of a second generation mutant screen utilizing a double transgene approach.

The analysis of plant mutants with altered vacuolar protein sorting will permit the identification as well as the characterization of both sorting signal receptors and components of the sorting apparatus that will be unique to plants or of broader significance.

RESULTS

Transformation and Analysis of *wt* and *ctpp*- Constructs in *Arabidopsis thaliana*.

The development of a genetic screen in *Arabidopsis* for vacuolar protein sorting mutants requires the presence of a vacuolar marker protein. However there are no known endogenous markers for the vacuole in *Arabidopsis*. The gramineae barley lectin (BL) is a homodimeric vacuolar protein that specifically binds the sugar *N*-acetylglucosamine (for review, see Raikhel and Lerner, 1991). BL is synthesized as a preproprotein with a high-mannose glycosylated CTPP that is removed before or concomitant with deposition of the mature protein into the vacuole. We have previously demonstrated that BL is correctly assembled, processed and targeted to the vacuole of transgenic tobacco (Wilkins et al., 1990). Deletion of the CTPP caused BL to be secreted from the cell (Bednarek et al., 1990). Other proteins containing CTPP vacuolar sorting signals, such as chitinases and glucanases have antigenically cross-reactive vacuolar and secreted isoforms that are present in many plants (Bednarek and Raikhel, 1992). Therefore, in order to use BL as the marker protein, it was necessary to determine if BL is correctly processed and targeted to the vacuole in *Arabidopsis*.

Arabidopsis thaliana (ecotype RLD) root explants were transformed with *Agrobacterium tumefaciens* containing pGA643 BL constructs *wt* (BL + CTPP) (Bednarek et al., 1990) and *ctpp*- (BL with CTPP deleted) (Wilkins et al., 1990)

as described in Valvekens et al., (1988). Kanamycin-resistant plants were isolated and screened for the production of BL by protein blot analysis as described in Wilkins et al., (1990) (data not shown).

Selected *wt* and *ctpp-* BL transformed plants were selfed 3 times. Southern blot analysis of T_3 generation plants, using a BL specific probe, indicated these plants contained one copy of the T-DNA insert at a single locus (Sebastian Bednarek, personal communication). The final selected transgenic lines *wt 5* and *ctpp- 3* expressing the BL constructs were then back-crossed to a wild type plant, to determine whether they were homozygous for the T-DNA insert. ALL F_1 progeny tested displayed kanamycin resistance as well as BL protein production, indicating the parental was homozygous for the BL locus.

Pulse-chase analysis and electron microscopy (EM) immunolocalization were used to determine if *wt* and *ctpp-* were correctly processed and targeted to the vacuole in *Arabidopsis.* Protoplasts prepared from the *wt 5* and *ctpp- 3* BL transformed *Arabidopsis*, were pulse labeled with a mixture of ^{35}S methionine and ^{35}S cysteine for 6 hours and chased for an additional 12 hours with unlabeled methionine and cysteine. Radiolabeled BL was affinity purified from crude protein extracts of protoplast and incubation medium at specified time points, and analyzed by SDS-PAGE and fluorography. The 23-kD polypeptide (proform) and mature 18-kD subunits of BL were readily discernable. During the 12 hour chase of *wt* (BL + CTPP) the disappearance of 23-kD proBL was accompanied by a corresponding increase in the level of the intracellular 18-kD mature subunit, while no appreciable accumulation of

radiolabeled BL in the corresponding media samples was observed (Figure 4.1). The faint bands observed in the BL + CTPP media are most likely due to cellular breakage during the course of the chase. However the 18-kD mature polypeptide of *ctpp-* (BL Δ CTPP) was detected in both the intracellular and incubation media fractions. During the 12 hour chase there was a decrease in the level of intracellular 18-kD polypeptide and a corresponding increase in the amount of the 18-kD BL subunit in the medium (Figure 4.1, BL Δ CTPP). We have also noted a slight decrease in the intensity of the 12 hour chase *ctpp-* media fraction when compared to the 0 hour intracellular time point. This loss of signal is most likely due to protein absorption by the polystyrene tissue culture plates used to incubate the protoplasts during labeling and not due to degradation. In addition, after the 12 hour chase a small amount of radiolabeled BL 23-kD (BL + CTPP) and 18-kD (BL Δ CTPP) remained associated with the protoplast fractions. These observations are discussed in Dombrowski et al., (1993). Furthermore, radiolabeled BL proteins were isolated using their ability to bind to an immobilized *N*-acetylglucosamine affinity column (Figure 4.1), indicating that the BL polypeptides were folding and dimerizing correctly (Wright, 1987; Mansfield et al., 1988; Schroeder and Raikhel, 1992; Wright et al., 1993).

Subcellular localization of the *wt* BL construct in transgenic *Arabidopsis* plants, by electron microscopic (EM) immunocytochemistry, localized BL to the vacuoles of roots and leaves, as shown in Figure 4.2A and 4.2C. The pulse-chase analysis (discussed above) of Arabidopsis plants expressing *ctpp-* BL

Figure 4.1. Pulse-Chase Labeling Experiments of Transgenic Protoplasts Expressing BL-Barley Lectin Constructs.

Pulse-chase labeling of protoplasts isolated from transgenic *Arabidopsis* plants expressing the *wt 5* wild type BL + CTPP and *ctpp- 3* mutant BLΔCTPP (total deletion of CTPP) constructs and corresponding incubation medium. Protoplasts were pulse labeled for 6 hr and chased for 12 hr. Protein extracts were prepared from the protoplasts and incubation media at specified time intervals (hr) as indicated during the chase. Radiolabeled BL was affinity purified and analyzed by SDS-PAGE and fluorography. The molecular mass of the 23-kD proBL subunit and mature 18-kD subunit of BL is shown to the left of the gels.

Pulse-Chase Analysis of *Arabidopsis* Transformed with Barley Lectin Constructs

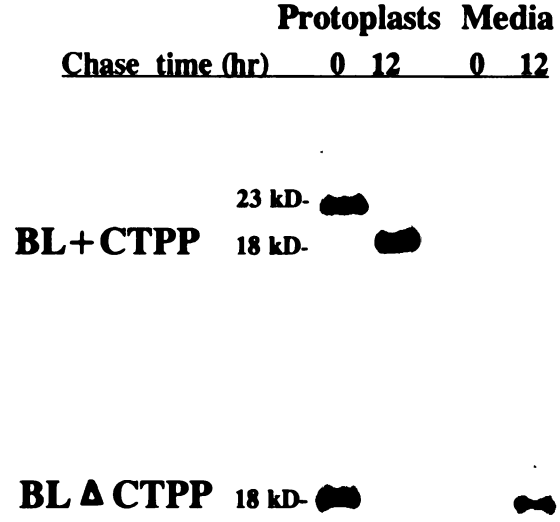
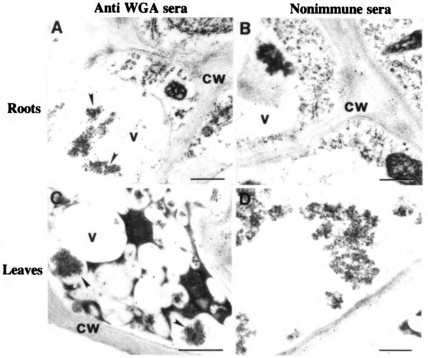


Figure 4.2 Immunocytochemical Localization of BL + CTPP construct.

Thin sections of transgenic line *wt 5, Arabidopsis* roots **(A)** and **(B)** and leaves **(C)** and **(D)** expressing BLΔCTPP constructs. **(A)** and **(C)** are treated with rabbit polyclonal anti-WGA antisera; **(B)** and **(D)** are treated with nonimmune sera. Gold labeling (arrow) in **(A)** and **(C)** is found exclusively in the vacuole of tobacco plants transformed with BL + CTPP. Bound antibodies were visualized with protein A coupled to 15 nm colloidal gold. Bar = 0.5 μm. CW, cell wall; V, vacuole.

Immunocytochemical Analysis of *Arabidopsis* Transformed with Barley Lectin +CTPP



construct (total deletion of the CTPP) showed BL is secreted from the cell, yet after a 12 hour chase a small amount of the protein remained associated with the protoplasts (Figure 4.1, BL Δ CTPP). However, as shown in Figure 4.3A and 4.3C, the EM immunocytochemical analysis of *ctpp-* transformed *Arabidopsis* plants showed no detectable labeling in the vacuoles. In *ctpp-* transformed plants BL was localized to the middle lamella of root and leaf cells. No specific labeling was detected in parallel experiments using nonimmune serum for *wt* and *ctpp-* transformed plants (Figures 4.2 B&D, 4.3 B&D).

Therefore, the analysis of the BL constructs in *Arabidopsis* demonstrated that BL + CTPP is correctly processed and targeted to the vacuole (Wilkins et al., 1990; Bednarek et al., 1990; Dombrowski et al., 1993).

Mutant Screen and Analysis

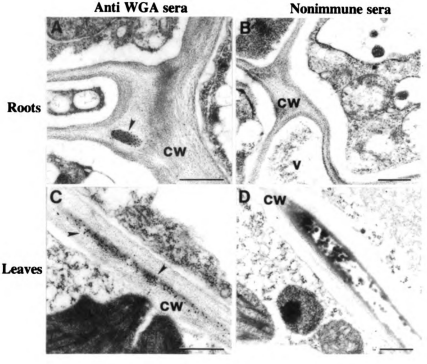
The main objective of this research was to establish a reliable screen and to identify mutant plants that show altered sorting of BL + CTPP. Homozygous seeds from a transgenic line *wt 5* expressing BL + CTPP were ethylmethane sulfonate (EMS) mutagenized by Lehle seeds (Tucson, AZ). After mutagenesis the seeds were germinated and the population size was estimated to be 31,902 M_1 parents divided into 26 parental groups (designated A-Z). The mutation frequency of albino embryo mutations in a random sample of M_1 siliques was calculated to be Mednik's P-value = 0.5 (Mednik, 1988). This would indicate a high mutation rate.

The initial screen analyzed the intercellular (apoplastic) wash fluid (ICWF)

Figure 4.3 Immunocytochemical Localization of BLΔCTPP construct.

Thin sections of transgenic line *ctpp- 3*, *Arabidopsis* roots **(A)** and **(B)** and leaves **(C)** and **(D)** expressing BLΔCTPP constructs. **(A)** and **(C)** are treated with rabbit polyclonal anti-WGA antisera; **(B)** and **(D)** are treated with nonimmune sera. Gold labeling (arrow) in **(A)** and **(C)** is found exclusively in the cell wall of *Arabidopsis* plants transformed with BLΔCTPP construct. Bound antibodies were visualized with protein A coupled to 15 nm colloidal gold. Bar = 0.5 μm. CW, cell wall; V, vacuole.

Immunocytochemical Analysis of *Arabidopsis* Transformed with Barley Lectin △ CTPP



from the leaves of 6,100 mutagenized plants (M_2 generation) for the presence of secreted BL. Leaves were incubated in MES/NaCl buffer under vacuum, and the pressure then rapidly released and reapplied again for a short period of time. The leaves were removed, blotted dry, and gently centrifuged to extract apoplastic fluid. The resulting apoplastic fluid was dotted onto nitrocellulose membrane and probed using antibodies against wheat germ agglutinin (WGA), which is antigenically indistinguishable from BL (Lerner and Raikhel, 1989). ICWF from parent plants expressing wild-type BL (BL+CTPP) and from non-transformed plants were used as negative controls. ICWF from plants expressing BL construct *ctpp-* (total deletion of CTPP) was used as a positive control, because in these plants BL is secreted from the cell. The sensitivity of the screen was established by determining the maximum number of *wt* leaves that could be combined with one leaf from a *ctpp-* plant. A signal on the blot could be detected when the leaf of positive control plants was combined at a ratio of 1:30 with the leaves of negative control plants (data not shown). Therefore a single leaf from 20 individual mutagenized plants were combined for the initial screening. When a positive signal was obtained from the pool of 20, the plants were divided into groups of 10 and screened. If a positive signal occurred from one of the smaller groups, the remaining plants were screened individually. Eight putative mutants secreting BL were identified by protein dot blot analysis of ICWF (Table 4.1).

In order to determine if the mutations were homozygous, five of the putative mutants (*avs1* thru *avs5)* were selfed, and M_3 progeny were checked

Table 4.1 Summary of the putative mutants analyses.

PM : Putative Mutant

Plants are designated by their parental group (A-Z), set number (the group of 20 plants initially screened), and the individual plant number isolated from the set.

Dot Blot analysis: (+) indicates a positive signal for BL in ICWF.

P\C - Pulse-chase analysis: (-) indicates BL was not found in the incubation medium.

EM - Electron Microscopy Immunolocalization of BL. (-) indicates that BL was exclusively localized to the vacuole; (+) indicates labeling was found equally distributed between cell wall and vacuole; (±) indicates strong labeling in vacuole and weak labeling in cell wall.

M3 - Dot blot analyses of ICWF from M3 generation plants:

# of plants testing positive / total # of plants tested.

Table 4.1

PM	Group	Set	Plant	Dot Blot	P\C	EM	M3
avs1	A	119	1	+	-	-	6/11
avs2	A	159	1	+	-	±	2/12
avs3	A	159	4	+	-	-	12/12
avs4	A	120	3	+	-	±	10/10
avs5	C	37	6	+	-	+	12/12
avs6	C	39	7	+	-	ND	ND
avs7	D	33	5	+	ND	ND	ND
avs8	F	10	3	+	ND	ND	ND

for the presence of BL in the ICWF by dot blot analysis. All M_3 progeny from mutants *avs3, avs4* and *avs5* tested positive for the presence of BL, indicating that they were homozygous for the mutation, see table 4.1.

The putative mutants (*avs1* thru *avs6*) were selected for analysis by pulse-chase labeling, to verify if BL is missorted and secreted from the cell. Protoplasts were prepared from the M_3 progeny of the mutants, pulse labeled with a mixture of ^{35}S methionine and ^{35}S cysteine for 6 hours and chased for an additional 12 hours with unlabeled methionine and cysteine. In addition, another set of these isolated protoplasts were labeled continuously for 20 hours. Radiolabeled BL was affinity purified from crude protein extracts of protoplast and incubation medium at specified time points, and analyzed by SDS-PAGE and fluorography. The BL (affinity purified) distribution for all of the mutants tested was found to be identical to the *wt* control (negative) plant cells (data summarized in Table 4.1); each mutant displayed a similar pattern on SDS-PAGE gels as the *wt* control plant (see Figure 4.1, BL + CTPP), with only faint amounts of radiolabeled BL detected in the media fractions. This small amount of BL in the medium was most likely due to cellular breakage during the isolation step. When the mutants were compared to the *ctpp-* positive control (Figure 4.1, BLΔCTPP) no appreciable accumulation of BL was observed in the medium fractions from the mutant cells (data not shown). In addition, immunoprecipitation of these fractions also displayed similar bands as the controls. The isolated radiolabeled bands from mutant plant cells were identical to the control plant *wt* (BL + CTPP, negative control, intracellular) and the *ctpp-*

(BLΔCTPP, positive control, BL secreted) with the exception of the 23 kD proBL band which was absent (data not shown). A possible explanation for these results could be that the mutants mislocalize very small amounts of BL. Therefore, over the time period used for the pulse chase analysis, this small amount of mislocalized BL would not be detectable above the background. However in a whole plant, BL would be able to accumulate in the extracellular space over time, and therefore, be detected at steady state levels by dot blot of ICWF and by EM.

EM immunolocalization was performed on M_3 progeny from five putative mutant plants (*avs1* thru *avs5*), in order to verify the presence of BL in the cell wall or intercellular spaces (results summarized in table 4.1). EM analysis confirmed that in three of the mutants (*avs2*, *avs4* and *avs5)*, BL is localized to the vacuoles and cell wall. Mutant *avs5* displayed the strongest labeling in the cell wall (see Figure 4.4). As a result of the EM findings, the mutant *avs5* has been selected for further genetic analysis. Mutant *avs5* is currently being backcrossed to the *wt* parental to determine a pattern of inheritance as well as dominance.

Figure 4.4 Secretion of BL in Mutant *avs5*.

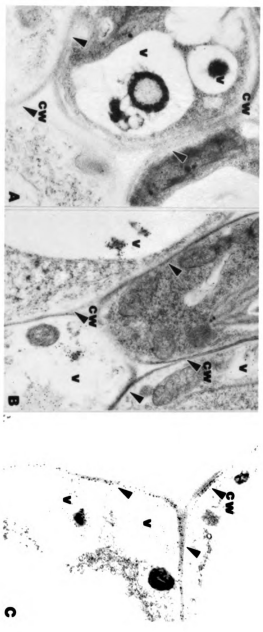
(A) Transgenic *Arabidopsis* expressing BL + CTPP, treated with preimmune sera.

(B) Transgenic *Arabidopsis* expressing BL + CTPP, treated with rabbit polyclonal anti-WGA sera. BL is localized to the vacuole with no labeling in the cell wall.

(C) EM immunolocalization of BL in the cell wall (arrows) and vacuole in mutant *avs5* from Parental Group C, Set #37, plant #6 [C37(6)].

Bound antibodies were visualized with protein A coupled to 10 nm colloidal gold.

Arrows denote location of cell wall. Bar = 5 nm. cw, cell wall; v, vacuole.


A
CW
V
CW
V
B
CW
V
CW
V
C
CW
V
V

DISCUSSION

The main focus of current research is to investigate the molecular mechanisms of protein sorting to the plant vacuole. Most soluble proteins are transported through the secretory system via a series of transport vesicles that bud from one compartment and fuse specifically with the next (for reviews see, Rothman and Orci, 1992; Pryer et al., 1992; Rothman, 1994). Three independent signals that direct proteins to plant vacuoles have been identified, NTPPs, CTPPs, and regions within mature proteins. There is no homology between these sorting signals, and all three signals appear to be unique to plants. In addition, recent experimental findings indicate that multiple receptors are involved in the targeting of soluble proteins to the vacuole (Kirsch et al., 1994), as well as different mechanisms for the transport of NTPP and CTPP containing proteins (Matsuoka et al., 1995). We have shown that when BL + CTPP is transformed into *Arabidopsis,* it is correctly processed and targeted to the vacuoles in roots and leaves. The creation of vacuolar protein sorting mutants in *Arabidopsis* should provide an excellent chance to isolate the CTPP receptor and other components of the sorting apparatus.

To create plant vacuolar sorting mutants, transgenic *Arabidopsis thaliana* plants expressing BL + CTPP were treated with EMS (Malmberg 1993; Feldmann et al., 1994). EMS was chosen because of the wide range of mutants found with this type of mutagenesis (Feldmann et al., 1994; Pepper et al., 1994).

Thus, we would expect to isolate null, conditional (temperature sensitive) and leaky mutations in the plant vacuolar sorting pathway. After successful EMS mutagenesis, the per-locus mutant frequency in the M_2 population is estimated in the range of 1 in 1000 to 1 in 5000, with the potential range of 2 to 75 additional mutations present elsewhere in the genome (Feldmann et al., 1994). The frequency of null mutations in a given gene under these conditions has been estimated at about 1 in 2,000 M_2 plants (Estelle and Somerville, 1986). The presence of the vacuole is believed to be essential for plant cell growth, and mutations in vacuolar protein sorting may severely disrupt the structure or inhibit the formation of the vacuoles. However, most yeast *vps* mutants are not lethal and display a wide range of vacuolar morphologies (Klionsky et al., 1990; Raymond et al., 1992 a,b). In addition, by using EMS mutagenesis, one can potentially create conditional (temperature sensitive) or leaky mutants which will allow for the isolation of essential genes (Feldmann et al., 1994).

Mutant Screen

After screening 6,100 M_2 plants, three putative mutants that secrete BL have been identified by dot blot analysis of ICWF and verified by EM immunolocalization. To verify that the positive signal is actually due to the presence of BL in the ICWF, the ICWFs of putative mutants should be passed over an affinity column of immobilized *N*-acetylglucosamine and analyzed by western protein blot analysis. If the mutants are secreting BL to the extracellular spaces, then the analysis of the eluted fractions from the affinity columns

should show enhanced levels of BL when compared to *wt* plants. However, if this analysis fails to show the presence of enhanced levels of BL, then the antigenic determinant recognized by the antibody could be a *cis*-mutation in BL. This mutation in BL could result in either its misfolding or a truncated version of the protein, both of which would be unable to bind to the *N*-acetylglucosamine affinity column. Furthermore, the mutation could have caused the production of a unrelated protein that contains a cross-reactive epitope.

One approach to determine whether the putative mutants are *cis* or *trans* in nature, is to cross the mutants with *Arabidopsis* plants expressing the vacuolar localized sporamin+CTPP construct (sporamin lacking a functional NTPP but containing BL's CTPP; Matsuoka et al., 1995). A *cis*-acting mutation would not affect the vacuolar localization of the sporamin+CTPP, whereas a *trans*-acting mutation would result in its secretion.

During the screening process many sickly plants were observed. With these plants it was extremely difficult, if not impossible, to obtain useable quantities of ICWF to analyze. Although many of the yeast *vps* null mutants are not lethal, they do affect growth. Furthermore, yeast is a unicellular organism and may tolerate mutations in vacuolar protein sorting. However, these same mutations in a multicellular plant may be more deleterious, disrupting growth and the development of tissues and higher order cellular structures. Therefore, mutations which affect plant vacuolar protein sorting may produce a sickly phenotype.

An evaluation of the initial genetic screen revealed a number of short comings, such as an inability to quickly distinguish between *cis* and *trans* mutations and not being able to analyze sickly plants. In addition, the screen was labor intensive. Therefore, a second generation screen is currently being developed to address these concerns.

Second Generation Screen

During the development of the preliminary genetic screen the only available soluble vacuolar reporter protein utilizing a CTPP which could be easily assayed for was BL+CTPP. Other proteins containing CTPP vacuolar sorting signals, such as chitinases and glucanases have antigenically cross-reactive and enzymatically indistinguishable vacuolar and secreted isoforms that are present in many plants (Bednarek and Raikhel, 1992; Raikhel et al., 1993; Collinge et al., 1993; Melchers et al., 1993). However, having only one reporter makes the screening for mutants considerably more difficult, not only in distinguishing *cis* from *trans* mutations, but also differentiating between legitimate vacuolar sorting mutants and potential false positives. Recently a number of potential vacuolar reporter proteins have been made available to us. In collaboration with Dr. Ken Matsuoka, it has been shown that sporamin (with a non-functional mutated NTPP) was correctly targeted to the vacuole of tobacco cells using the CTPP of BL (Matsuoka et al., 1995). In addition, at the 1995 Keystone meeting on Plant Cell Biology, Dr. Jean-Marc Neuhaus presented data showing that when the 7 amino acid CTPP of tobacco chitinase is fused to the C-terminus of

rat beta-glucuronidase (RGUS) (Nishimura et al., 1986; Powell et al., 1988), this chimeric protein was efficiently targeted to the vacuoles in tobacco (Chrispeels et al., 1995). In addition, RGUS has a 15 amino acid C-terminal extension that will partially direct RGUS to the plant vacuole. The deletion of this extension from the protein causes its total secretion from the cell (personal communication, Dr. Jean-Marc Neuhaus). Therefore, the ability of RGUS to be directed to the vacuole will allow for the development of a colorimetric assay to screen for vacuolar sorting mutants. The development of this screen in *Arabidopsis* is currently underway. The screen will select for mutants whose roots are missorting and secreting the normally vacuolar localized RGUS + CTPP. A small number of seeds of transgenic plants expressing the secreted RGUSΔ15 (deletion of C-terminal extension) will be mixed with a large number of seeds from plants expressing the vacuolar RGUS + T (RGUS with CTPP of tobacco chitinase). After the mixed seeds have germinated on agar plates, their roots will be overlaid with a substrate that will allow for color development if RGUS activity is present. Assay conditions will be altered until the small number of RGUSΔ15 seedlings can be distinguished from the RGUS + T seedlings. The development of this assay would dramatically decrease the time and labor involve in screening for mutants by eliminating the necessity for the isolation of ICWF. Furthermore, this method will allow for the screening of sickly plants.

The availability of these second CTPP-containing reporter proteins permits the design of a double-transgene approach (see Figure 4.5), that will avoid the necessity of genetic crosses in the initial screen to differentiate between *cis* and

trans mutations. In addition, the presence of two reporter proteins will provide a means by which mutants can be discriminated from false positives by two different determinants. Specifically, the binary vector pMOG800 containing BL + CTPP and RGUS + T (Figure 4.5A) or sporamin + CTPP (Figure 4.5B) genes, each driven by a double 35S promoter and terminated with the potato proteinase inhibitor terminator (pMOG843 cassette), will be introduced into *Arabidopsis*. The complementary constructs with CTPP's deleted will also be made for positive controls (Figures 4.5D & 4.5E). There are multiple examples of using more than one reporter gene in the same vector. For example, a very similar test/reference gene system was used in analysis of RNA degradation in plants (Newman et al., 1993; Ohme-Takagi et al., 1993). In addition, the expression of multiple genes in plants utilizing the expression cassette of pMOG843 and delivered by the binary vector pMOG800 has been successfully accomplished by scientists at MOGEN International (Dr. Stephan Ohl, personal communication).

Since NTPP and CTPP containing proteins appear to use different receptors (Kirsch et al., 1994) as well as different mechanisms (Matsuoka et al., 1995) for their delivery to the vacuoles, the prediction would be that some mutants will specifically missort NTPP-containing proteins. Recently obtained data showed that the NTPP of sporamin can function as a vacuolar sorting signal for BL (BL without the CTPP but fused at the amino-terminus to sporamin's NTPP, Matsuoka et al., 1995). Therefore, an additional mutant screen will be performed on *Arabidopsis* plants transformed with a double transgene (Figure

Figure 4.5 Schematic Representation of the Double Transgene Expression Cassettes in the Binary Vector pMOG800.

Kanamycin resistance: nopaline synthase promoter (nos):: neomycin phosphotransferase II (NPTII):: nos terminator (Angenon et al., 1994).

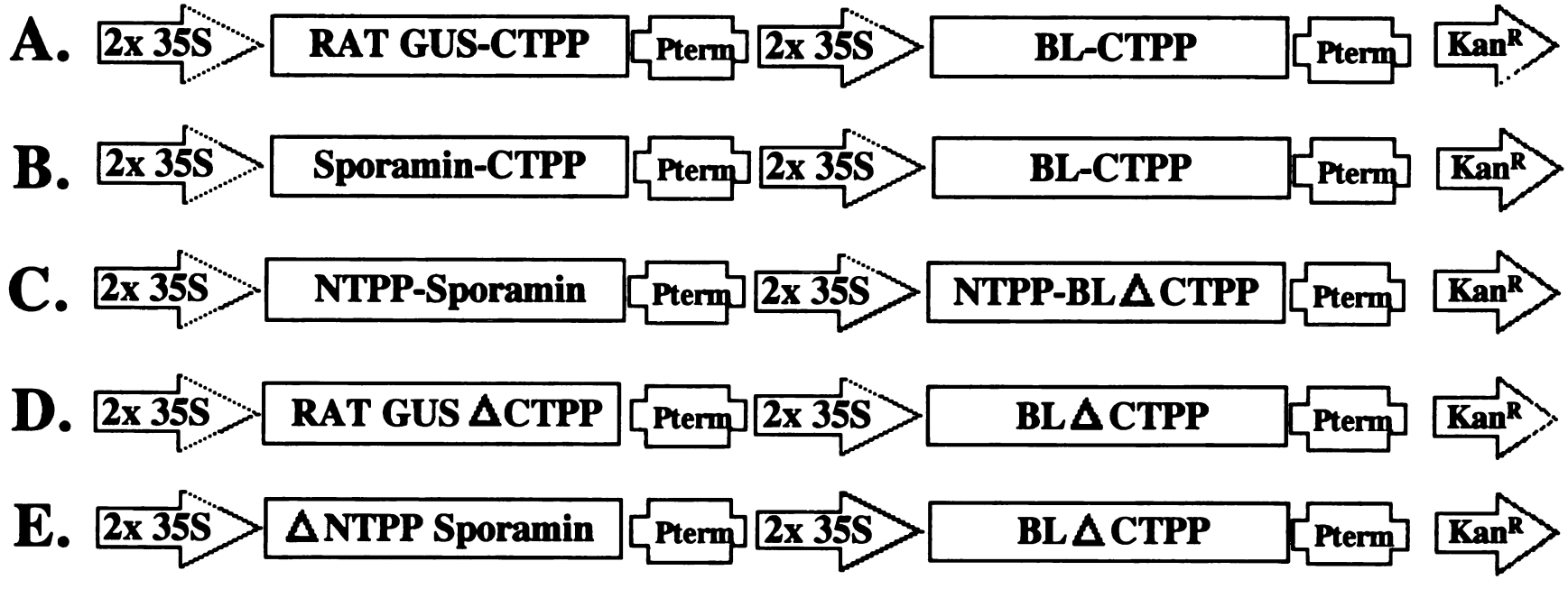
(A). Contains CDNA constructs; the chimeric Rat GUS with the 7 amino acid (-GLLVDTM) CTPP of tobacco chitinase (Neuhaus et al., 1994) fused to its 15 amino acid C-terminal extension (RGUS-SVPRTQCMGSRPFTF + GLLVDTM) and the wild type BL + CTPP clone.

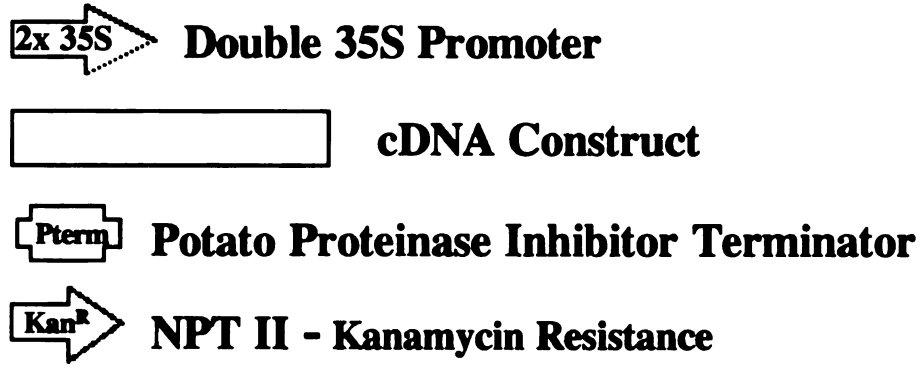
(B). Contains CDNA constructs; the chimeric I28G-NTPP-sporamin + CTPP of BL (Sporamin-VFAEAIAANSTLVAE) (I28G: Isoleucine^{28} is substituted with Glycine, inactivating the targeting signal Nakamura et al., 1993) and the wild type BL + CTPP clone.

(C). Contains CDNA constructs; the wild type NTPP + sporamin and the NTPP of Sporamin fused to BLΔCTPP (deletion of its 15 amino acid CTPP).

(D). Contains CDNA constructs; the Rat GUSΔ15 (deletion of its 15 amino acid C-terminal extension) and BLΔCTPP (deletion of its 15 amino acid CTPP).

(E). Contains CDNA constructs; ΔNTPP-sporamin (deletion of its NTPP) and BLΔCTPP (deletion of its 15 amino acid CTPP).


A.
2x 35S
RAT GUS-CTPP
Pterm
2x 35S
BL-CTPP
Pterm
Kan^R
B.
2x 35S
Sporamin-CTPP
Pterm
2x 35S
BL-CTPP
Pterm
Kan^R
C.
2x 35S
NTPP-Sporamin
Pterm
2x 35S
NTPP-BLΔCTPP
Pterm
Kan^R
D.
2x 35S
RAT GUS ΔCTPP
Pterm
2x 35S
BLΔCTPP
Pterm
Kan^R
E.
2x 35S
ΔNTPP Sporamin
Pterm
2x 35S
BLΔCTPP
Pterm
Kan^R


2x 35S
Double 35S Promoter
cDNA Construct
Pterm
Potato Proteinase Inhibitor Terminator
Kan^R
NPT II - Kanamycin Resistance

4.5C) expressing NTPP-containing proteins which have been mutagenized with EMS (Figure 4.5C).

Therefore, homozygous plants containing both genes at a single locus will be isolated and their seed subjected to EMS mutagenesis. M_2 plants will be screened using the dot blot method for secretion of both BL and sporamin, or in combination with the RGUS colorimetric assay described above. This screen will be done under conditions which will allow for the identification of those mutants which are temperature sensitive. By selecting for conditional mutants it may be possible to isolate mutants which might otherwise be lethal to the plant. Additionally, mutants will be analyzed morphologically by EM to determine whether any structural alterations in the vacuole have occurred. Mutants which have altered vacuolar morphologies may also provide valuable information on the process of vacuole biogenesis. The remainder of the genetic approach used to analyze the mutants, as well as the overall general scheme for the entire screening process for both CTPP and NTPP containing proteins is shown in Figure 4.6.

The long-term goal of this project is to identify and isolate components of the vacuolar sorting machinery, and to elucidate their role in transport to the vacuole. The development of the second generation screen for the isolation of vacuolar sorting mutants discussed above will provide the means by which this goal can be achieved. The isolation of mutants which specifically affect the sorting of either CTPP or NTPP-containing proteins will have the highest potential to identify components that are unique to plants, such as receptors,

and will provide important insights into the fundamental processes of plant vacuolar sorting.

Figure 4.6 General Scheme for the Creation and Isolation of Vacuolar Sorting Mutants in *Arabidopsis*.

Double transgene approach see Figure 4.5.

RGUS-CTPP-> BL-CTPP-> Figure 4.5A; <-BL-NTPP-<-SPO-NTPP Figure 4.5C.

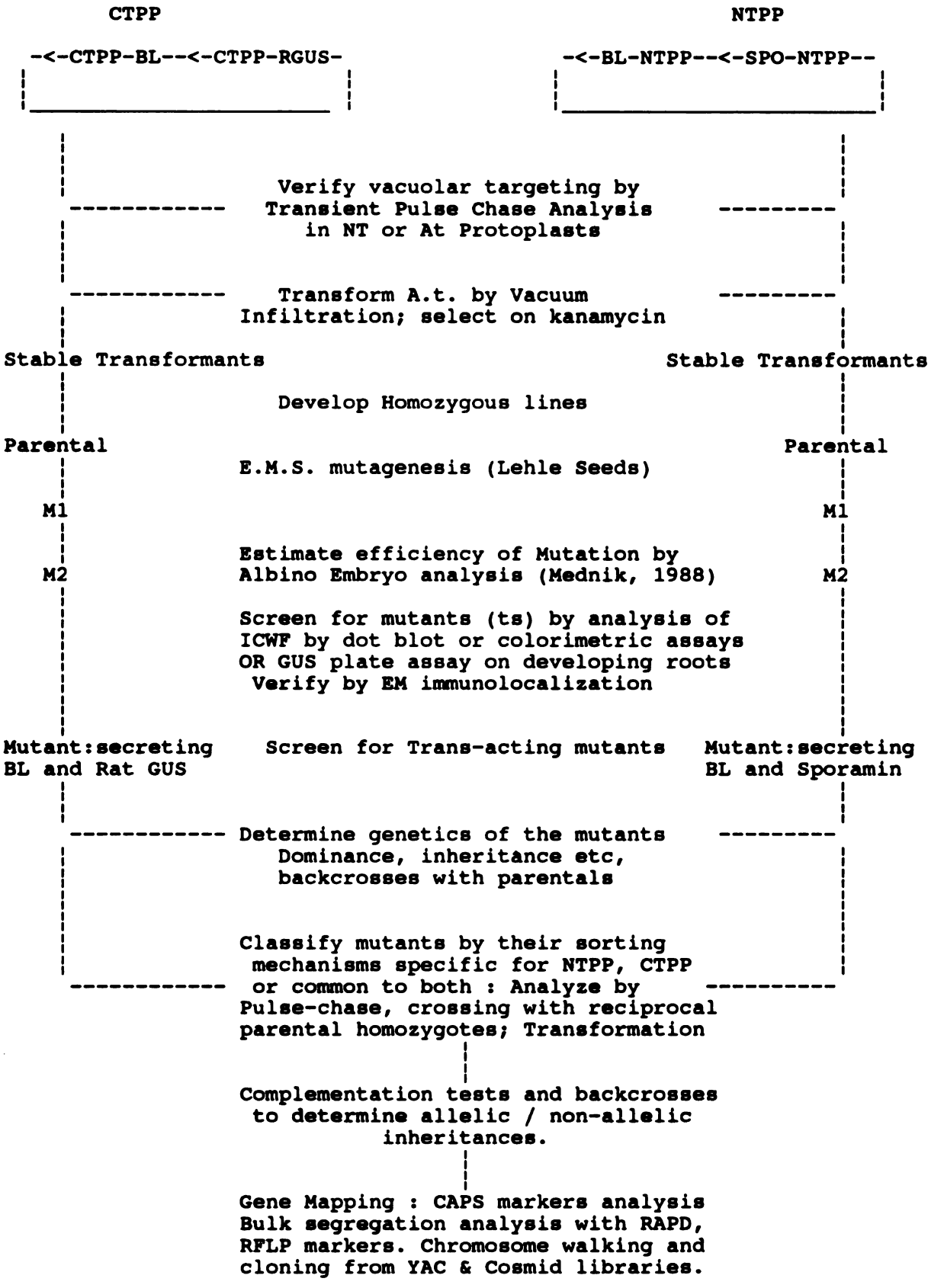
NT-*Nicotiana tabacum* or At-*Arabidopsis thaliana*

Codominant cleaved amplified polymorphic sequences (CAPS) markers (Konieczny and Ausubel, 1993).

Random amplification of polymorphic DNA (RAPD) and restriction fragment length polymorphisms (RFLP) markers (Michelmore et al., 1991; Reiter et al., 1992; Rafalski et al., 1994; for a review on *Arabidopsis* genetics see, Koornneef, 1994).

Yeast artificial chromosome (YAC) and cosmid libraries (Ward and Jen, 1990; Grill and Somerville, 1991; Meyerowitz, 1994; Scholl et al., 1994).

Vacuolar Protein Sorting Mutant Screen & Analysis



MATERIALS AND METHODS

All standard recombinant DNA procedures used in this study were carried out as described in Sambrook et al. (1989), unless otherwise noted. DNA restriction and modifying enzymes were obtained from Beohringer Mannheim (Indianapolis, IN), Gibco-BRL (Gaithersburg, MD), or New England BioLabs (Beverly, MA). All other reagents, unless specified, were purchased from Sigma. The *E. coli* strain DH5α was used for all DNA manipulations unless otherwise noted.

Preparation of Constructs

The barley lectin (BL) constructs used for *Arabidopsis* transformation in the preliminary genetic screen were the wild type BL (*wt*) (Wilkins et al.,1990), and BL with the 15 amino acid CTPP deleted (*ctpp*-)(Bednarek et al., 1990). These barley lectin cDNA constructs are in the binary plant expression vector pGA643 (An et al., 1988) and contained in *Agrobacterium tumefaciens* LBA4404.

The cloning strategies used for the construction of the double transgene cassettes for the second generation screen were as followed. The cloning vector pBS SK- (Stratagene, Lajolla Calif.) was cut with EcoRV and HincII and then religated, in order to eliminate the HindIII restriction site. This plasmid was designated pBS SK E/H. The plant expression vector pMOG 843 (MOGEN International, Netherlands), is an ampicillin resistant plasmid, which contains the cassette of a double 35S promoter: HindIII and KpnI restriction sites: potato

proteinase inhibitor terminator, flanked by XbaI restriction sites. The pMOG843 was cut with KpnI, blunt ended with T4 DNA polymerase (Boehringer Mannheim), and religated; in order to eliminate the KpnI restriction site, designated pMOG843 XK. The plasmid pMOG843 XK was then cut with XbaI to drop out the modified cassette, and was cloned into the XbaI site in pBS SK E/H. Plasmids were isolated for both orientations of the cassette insertion. They were designated pBS843 XKEH #10 (with the orientation in the polylinker, SacII:2x35S:HindIII:Pot Term:XhoI,KpnI) and pBS843 XKEH #14 (SacII:Pot Term:HindIII:2x35S:XhoI,KpnI). Both plasmids were cut with HindIII, blunt ended with T4 DNA polymerase, and treated with calf intestine alkaline phosphatase.

The pUC118 plasmids containing the barley lectin (BL) cDNA constructs wild type BL (*wt*) (Wilkins et al.,1990), and BL with the 15 amino acid CTPP deleted (*ctpp*-)(Bednarek et al., 1990) were excised with EcoRI, blunt ended with T4 DNA polymerase, and ligated into pBS843 XKEH #10; designated pBS843 BL, and pBS843 CTPP-.

The plasmids pMAT 110 (NTPP-Sporamin, Matsuoka and Nakamura, 1991), pMAT108 (deletion of NTPP from Sporamin, Matsuoka and Nakamura, 1991), and pMAT264 (mutated NTPP of sporamin+CTPP of BL, Matsuoka et al.,1995) were cut with PstI and HindIII to release the cDNA fragment, the fragment blunt ended with T4 DNA polymerase, and ligated into pBS843 XKEH #14; designated pBS843 110, pBS843 108 and pBS843 264 respectively.

The plasmid pMAT 196 (NTPP-BL with CTPP deleted, Matsuoka et al.,

1995), was cut with KpnI and SalI, the resulting cDNA fragment blunt ended with T4 DNA polymerase, and ligated into pBS843 XKEH #10; designated pBS843 196.

The plasmids pRGUS+T (coding for Rat β-glucuronidase cDNA+CTPP of tobacco chitinase, Jean-Marc Neuhaus personal communication) and pRGUSΔ15 (coding for Rat β-glucuronidase cDNA with the carboxy-terminal 15 amino acids deleted, Jean-Marc Neuhaus personal communication), the cDNAs are in the vector pGY1 (Neuhaus et al.,1994), were cut with PstI and BamHI to release the cDNAs, the resulting cDNA fragments blunt ended with T4 DNA polymerase, and ligated into pBS843 XKEH #10 vector; designated pBS843 RGT and pBS843 RGΔ15. All pBS843 constructs will be checked for expression by transient assays in tobacco or *Arabidopsis* protoplasts.

The binary vector pMOG800 (MOGEN International), utilizing kanamycin resistance (nos promoter:NPTII:nos terminator), was cut with the restriction enzymes XhoI and KpnI. Subcloning of the expression cassettes described above to generate the double transgene constructs were as followed.

The plasmid pBS843 BL was cut with KpnI and SacII, the plasmid pBS843 RGT was cut with XhoI and SacII, and the two isolated fragments ligated into the precut binary vector pMOG800, see Figure 4.5A.

The plasmid pBS843 BL was cut with KpnI and SacII, the plasmid pBS843 264 was cut with XhoI and SacII, and the two isolated fragments ligated into the precut binary vector pMOG800, see Figure 4.5B.

The plasmid pBS843 196 was cut with KpnI and SacII, the plasmid pBS843

110 was cut with XhoI and SacII, and the two isolated fragments ligated into the precut binary vector pMOG800, see Figure 4.5C.

The plasmid pBS843 CTPP- was cut with KpnI and SacII, the plasmid pBS843 RGΔ15 was cut with XhoI and SacII, and the two isolated fragments ligated into the precut binary vector pMOG800, see Figure 4.5D.

The plasmid pBS843 CTPP- was cut with KpnI and SacII, the plasmid pBS843 108 was cut with XhoI and SacII, and the two isolated fragments ligated into the precut binary vector pMOG800, see Figure 4.5E.

These second generation constructs (Figure 4.5) in the binary vector pMOG 800 will be introduced into *Agrobacterium* GV3101 by electroporation (Walkerpeach and Velten, 1994).

Plant Growth and Maintenance

Seeds of *Arabidopsis thaliana* (ecotypes RLD and Col-0) were sterilized using 1.75% sodium hypochlorite, 0.1% triton X-100 with continual mixing for 20 mins, the seeds are washed 6 times in sterile water for 10 mins per wash, and are plated on Germination Medium (GM) 0.8% phytoagar plates (4.3 g/L MS salts (Murashige and Skoog (1962) (Gibco-BRL), 0.5 g/L MES, 10 g sucrose, 0.1 g/L myo-inositol, 1 mg/L thiamine-HCl, 0.5 mg/L pyridoxine and 0.5 mg/L nicotinic acid, adjusted to pH 5.7 with KOH), if using transformed seed then GM is supplemented with the appropriate antibiotic (generally kanamycin 50 μg/mL). The plates are then cold treated for 24 hours, 4°C, and the seeds germinated under constant light approx. 150 micro einsteins/m^2,

24°C. After germination the seedlings are placed under 16 hr light, 8 hr dark cycle. Seedlings can then be used or transferred to soil.

Soil used to grow plants will have the following composition, 1:1:1 Bacto potting soil (Michigan Peat Company): 3# vermiculite : coarse perlite, presoaked with either a nutrient solution as described by Somerville and Orgen, (1982), or with Peter's professional plant food all purpose 20:20:20 mix (Grace Sierra Horticultural Products, Millpitas Calif.). In the mutant screen the plants were grown at 5 plants per pot.

Plant Transformation

Arabidopsis thaliana root explants (ecotype RLD) were transformed essentially as described by Valvekens et al., (1988) using the binary vector pGA643 (An et al., 1988) containing the *wt* (Wilkins et al.,1990), and *BL ctpp-* (Bednarek et al., 1990) barley lectin cDNA constructs in *Agrobacterium tumefaciens* LBA4404. The plants were screen for expression of BL constructs and analyzed as the tobacco plants described in Wilkins et al. (1990) and Bednarek et al., (1990).

Constructs for the second generation genetic screen will be transformed into *Arabidopsis thaliana* (ecotype Col-0) by vacuum infiltration (Bechtold et al., 1993). Seeds are planted in 3.5"pots (9-12 plants/pot) which have been presaturated with nutrient solution and cover with a nylon mesh. Cold treated at 4°C for 24-28 hrs. Grow plants to a stage where bolts are just emerging, clip off bolts to encourage growth of multiple secondary bolts. Infiltration will be

done 4-8 days after clipping. There should be a large number of little inflorescences that are just emerging from the rosette, as well as young unopened floral buds, with a few bolts that are 10 cm tall but the majority in the 1-5 cm range. Use three pots per construct for each transformation.

Grow 1 liter overnight Luria Broth culture of *Agrobacterium* GV3101 harboring the binary vector pMOG 800 containing the construct of interest with antibiotic selection gentimycin 25ug/mL, kanamycin 25ug/mL. Harvest cells by centrifugation 4100xgs, 10 min, at RT (OD_{600} approx 0.8) and resuspend in 600 mLs of infiltration medium (2.15 g/L MS salts (Gibco-BRL), 3.19 g/L Gamborg's B5 basal medium with minimal organics (Sigma), 50 g/L sucrose, and 0.044 μM benzylamino purine). The resuspended *Agrobacterium* is transferred to a 12" vacuum desiccation jar. Note pots should be fairly dry, turn pots on their side and water the very top soil of the pots. Invert plants into the suspension, give a small twist to submerge flower parts completely. The level of the *Agrobacterium* suspension should be right up to the mesh and leaves but should not enter the pot or soil. Place the contents under a strong vacuum with pump until rapid bubbling about 1-2 minutes, then release vacuum very rapidly, leave plants in suspension for 3-4 minutes. Remove plants from chamber and lay them on their side in a plastic flat to drain, keep separate the various construct with wax paper, cover with plastic wrap or preferably a dome to maintain humidity. Next day, uncover plants and set upright in growth chamber. Note do not water plants until pots are almost dry (4-7 days). Grow approximately 4 weeks, collect seed, sterilize and germinate on selection 0.8% agar plates (2.15

g/L MS salts [Gibco-BRL], 3.19 g/L Gamborg's B5 basal medium with minimal organics [Sigma], supplemented with 50 ug/ml kanamycin), select transformats and analyze for expression of the gene(s) of interest.

Mutagenesis by LEHLE SEEDS.

1.625 grams of BL transgenic seeds were surfaced sterilized by soaking in water for 30 min, then 95% EtOH for 5 mins, followed by 10% Chlorox bleach for 5 mins. The seeds were washed 5 times with water, then treated with 0.2% (v/v) ethylmethane sulfonate for 12 hours. The seeds were then rinsed 15 times with water over a 4 hour period. The seeds mutation batch 93B, were then germinated in 26 different flats containing 1:1:1:1 ratio of vermiculite : perlite : peat moss : Fisons Sunshine All purpose mix. M1 population size was estimated by taking a stand count of the emerged M1 seedlings. The stand count estimation is performed by randomly placing a 4.2 x4.8 cm metal wire frame on each flat of plants and counting the number of plants that fell within the frame boundary. This measurement was repeated 3 times per flat, and then the an average number of plants per square centimeter was calculated, and multiplied by the total number of square centimeters used for their growth. Estimated M1 population size was 31,902 M1 parents divided into 26 parental groups.

Immunocytochemistry

Electron microscopy immunocytochemistry was performed on individually expressing BL constructs or selected mutagenized BL transformed *Arabidopsis thaliana* plants essentially as described in Bednarek and Raikhel (1991). The primary antibody was rabbit anti-WGA antiserum (Raikhel et al., 1984) diluted, 1 to 20 or 1 to 50, and control sections were incubated with nonimmune serum diluted similarly. Protein A-colloidal gold (EY Laboratories Inc., San Mateo, CA) was diluted 1 to 50.

Vacuum Infiltration of *Arabidopsis* Leaves

Plants were germinated on GM 0.8% agar plates supplemented with kanamycin 50 μg/mL, and seedlings transferred to soil after 2 weeks. One leaf is then cut from 20 individual M2 plants. The leaves placed together wrapped in cheesecloth, in a side-armed erlenmeyer flask containing enough Infiltration solution (50 mM 2-[*N*-morpholino]ethanesulpfonic acid [MES], 100 mM NaCl) to submerge the tissue (200 mLs). The tissue is subjected to 15 in Hg vacuum, for 10 mins, break vacuum quickly, reintroduce vacuum an additional 10 mins. Intercellular wash fluid (ICWF) is obtained by centrifugation 1800xgs, 10 min at 4°C.

Western Dot Blot Analysis of ICWF

For immunoblot analysis, 4μl ICWF dotted on nitrocellulose membranes, air dried, and blocked for a minimum of 2 hrs with TBS (20 mM Tris-HCl, pH

7.4, 150 mM NaCl) containing 5% (w/v) non-fat dry milk. The membranes were incubated for 1.5 hr with WGA antiserum diluted 1:2000 in TBS containing 1% (w/v) BSA and 0.05% (v/v) Tween-20. After washing in TBS-0.1% (v/v) Tween-20, membranes were incubated for 1 hr with goat anti-rabbit antibody conjugated to alkaline phosphatase (Kirkegaard and Perry Lab Inc., Gaithersburg MA) diluted 1:7500 in TBS containing 1.0% (w/v) BSA and 0.05% (v/v) Tween-20. Secondary antibody binding was visualized as described by Blake et al. (1984).

Isolation and Radiolabeling of Transformed *Arabidopsis* Leaf Protoplasts

Protoplasts were prepared from 2-3 week old *Arabidopsis thaliana* seedlings (≈300 plantlets), which are germinated on GM Media 0.8% phytoagar plates (4.3 g/L MS salts (Gibco-BRL), 0.5 g/L MES, 10 g sucrose, 0.1 g/L myo-inositol, 1 mg/L thiamine-HCl, 0.5 mg/L pyridoxine and 0.5 mg/L nicotinic acid, adjusted to pH 5.7 with KOH) supplemented with 50 μg/ml kanamycin.

The leaves of whole seedlings are cut in 40 mls of 0.5 M betaine, 1.5 mM MES, adjusted pH to 5.7 with KOH, and incubated at room temperature (RT) for 45 minutes. The medium is then removed and the tissue is digested overnight in 50 mL enzyme mixture comprised of 1.5% cellulase (Onozuka R10), 0.5% macerozyme R10 (Yakult Honsha Co., Ltd Japan), and 0.08% BSA in 0.4 M betaine solution (0.4 M betaine, 10 mM $CaCl_2 \cdot 2H_2O$, 3 mM MES, pH 5.7). After 12-14 hrs the protoplasts are isolated essentially as described for whole leaf

tobacco protoplasts in Dombrowski et al., (1994), with the following changes. Protoplasts are washed in 0.4 M betaine solution, purified by flotation in 0.4 M sucrose solution (0.4 M sucrose, 10 mM $CaCl_2 \cdot 2H_2O$, 3 mM MES, pH 5.7), and washed twice in 0.4 M betaine solution. Viable protoplasts were visualized by fluorescein diacetate staining (Widholm, 1972) and the yields quantitated using a hemocytometer counting chamber. Protoplasts are washed again in 0.4 M betaine solution, and diluted to a final concentration of 500,000 or 1,000,000 protoplasts per milliliter in Incubation Medium (Gamborg's B5 basal medium with minimal organics (Sigma) supplemented with 0.3 M betaine, 0.1 M glucose, 0.15 mg/L benzyladenine, 1 mg/L 2,4-D, 0.08 g/L myo-inositol, 0.8 mg/L thiamine-HCl, 0.8 nM EDTA, adjusted to pH 5.7 with KOH). Then 1 ml of protoplasts are transferred to 12 well tissue culture plates (Costar, Cambridge, MA.) which have been precoated for 6 hrs with Incubation media supplemented with 1.5% BSA.

To examine expression of the barley lectin constructs, the transformed leaf protoplasts were incubated for 6 hr (pulse-chase analysis) or 20 hr (pulse-labeling) in the presence of 100 μCi Expre^{35}S^{35}S sulfur-35 protein labeling mixture (New England Nuclear Research Products), *E. coli* hydrolysate containing a mixture of 73% L-^{35}S-methionine and 22% L-^{35}S-cysteine in 50 mM tricine, 10 mM βME buffer (specific activity 1000-1100 Ci/mmol; ^{35}S-Met/Cys). If a pulse-chase analysis was performed after 6 hr of labeling, 100 μL of chase mix (165 mM methionine and 110 mM cysteine [free base] in Incubation Medium) was added and incubated for an additional 12 hr. After

labeling, the protoplasts were transferred to 1.5 ml microfuge tubes and separated from the culture medium by brief centrifugation (15-20 sec) at 800xg at room temperature.

If the samples are to be immunoprecipitated, the protoplast pellets were lysed in 500 μl of TNET250 (25 mM Tris-HCl, pH 7.5, 250 mM NaCl, 5 mM EDTA, 1% Triton X-100 [v/v]) (Firestone and Winguth, 1990) and cleared of insoluble debris by centrifugation at 16,000g for 5 min at 4°C. The extracellular protein fractions were prepared from the filtered incubation media as described in Bednarek and Raikhel, (1991) with 50 mg BSA added as nonspecific "carrier" protein. The culture medium/BSA protein precipitates were resuspended in 500 μl TNET250. For immunoprecipitation, 100 μl of 50 mg/ml BSA was added to the protoplast and media extracts.

If the samples are to be purified by affinity chromatography. The protoplast pellet was resuspended in 400 μL of extraction buffer, 50 mM Tris-acetate, pH 5.0, 100 mM NaCl, and 0.6% Triton X-100. The lysate was cleared of insoluble debris by centrifugation at 16,000g for 5 min at 4°C, frozen in liquid N_2, and stored at -70°C. The culture medium (1 mL) was filtered to remove any remaining protoplasts (Wilkins et al., 1990), and 25 μL of a 50 mg/mL BSA solution was added as a carrier protein. Proteins in the culture media were precipitated with ammonium sulfate at 70% saturation at 4°C for 2 hr then collected by centrifugation at 10,000 rpm for 10 min at 4°C. The culture medium protein pellet was resuspended in 400 μL extraction buffer and stored at -70°C. All protein samples were thawed at room temperature and

passed four times over immobilized *N*-acetylglucosamine (Pierce Chemical Co.) micro affinity columns (Mansfield et al., 1988). After extensive washing of the column with TA buffer (50 mM Tris-acetate, 100mM NaCl, pH 5.0), BL was eluted with 150 μL of 200 mM N-acetylglucosamine and lyophilized. The radiolabeled barley lectin was analyzed by SDS-PAGE through 12.5% polyacrylamide gels and visualized by fluorography as detailed in Mansfield et al. (1988).

Transient Gene Expression in Leaf Protoplasts

The transient expression of pBS843 constructs in tobacco leaf protoplasts via the PEG-mediated DNA uptake method was performed as described in (Dombrowski et al., 1993, 1994), and in *Arabidopsis* as described by Abel and Theologis (1994).

Immunoprecipitation and Analysis of Proteins

Sporamin and BL proteins were purified by immunoprecipitation as essentially as described by Schroeder et al., (1993) and Bednarek and Raikhel, (1991). To remove nonspecifically binding proteins, ^{35}S-labeled protoplast and media extracts were treated with 20 μl of nonimmune rabbit sera for 30 min at room temperature. Nonspecific protein immunocomplexes were reacted with fixed *Staphylococcus aureus* for 30 min at room temperature and removed by centrifugation at 16,000g for 5 min. Two microliters of anti-sporamin or 2.5 μL anti-WGA antiserum was added to the cleared extracts and incubated at

room temperature for 45 min. Immunocomplexes were collected on protein A-Sepharose CL-4B beads (Pharmacia, Piscataway NJ) for 30 min at room temperature and washed three times with TNET250 with continuous mixing for 5 min per wash. Bound proteins were released by heating at 95°C for 5 min in 30 μl of SDS-PAGE sample buffer. Samples were analyzed by SDS-PAGE on 12.5% polyacrylamide gels and visualized by fluorography as described previously (Mansfield et al., 1988).

Acknowledgments

We would like to thank Olga Borkhsenious for performing most of the initial screening and electron microscopy immunocytochemistry. Amy Sandul for her work with the *Arabidopsis* tissue culture. Sridhar Venkataraman for his help on Figure 4.6. Drs Stephan Ohl and Theo C. Verwoerd from MOGEN International for generously providing us with the plasmid for the expression vector pMOG843 and binary vector pMOG800. Dr. Ken Matsuoka for the plasmids pMAT108, pMAT110, pMAT264 and pMAT196. Dr Jean-Marc Neuhaus for the plasmids pRGUS+T and pRGUSΔ15 We would also like to thank all members of our laboratory for many helpful discussions. This research was supported by grants from the National Science Foundation, Washington, DC (Grant No. DCB-9002652) and the United States Department of Energy, Washington, DC (Grant No.DE-AC02-76ERO-1338) to N.V.R.

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CHAPTER 5

FUTURE RESEARCH

Currently, little is known about the mechanisms or machinery involved in targeting proteins to the plant cell vacuole. Conventional biochemical and molecular approaches have yielded limited success, identifying five potential components of the plant vacuolar sorting machinery (For a discussion see Chapter 1). However, the functions of these proteins have yet to be demonstrated in plants. One can attempt to demonstrate the function of a protein in plants by creating mutants through the suppression of their gene using an antisense approach. Once these gene products have been shown to be involved in vacuolar targeting, the proteins can be used to isolate other interactive components of the sorting apparatus. The isolation of additional components and the study of their interactions will impart a better understanding of the fundamental processes of protein transport in the cell.

The use of inhibitors such as monensin, Brefeldin A, tunicamycin and, most recently, wortmannin have shed some light on the mechanisms involved in protein transport to the vacuole (for a discussion see, Chapter 1). Through the use of inhibitors it was determined that vacuolar proteins are sorted at the *trans*-Golgi network (TGN), and that soluble proteins which use either amino-terminal propeptide (NTPP) or carboxyl-terminal propeptide (CTPP) targeting signals are delivered to the vacuole by two different mechanisms.

The analysis of the different plant targeting determinants has led to some surprising findings. Various NTPPs share a common motif within their sequences (see Chapter 1, Figure 1.1), and it is this specificity that allowed for

the isolation of a putative NTPP-receptor (Kirsh et al.,1994). However, in contrast to the specific nature of NTPP, CTPPs showed no consensus sequence or common structural determinants (see Chapter 1, Figure 1.2). In addition, correct vacuolar targeting was be achieved by a CTPP sequence as short as 3 exposed amino acids, and the interaction with the sorting apparatus appears to be at the C-terminus of the propeptide (Dombrowski et al., 1993). The question arises, why would a plant cell have a recognition system that displays such a broad binding specificity, as well as having two different sorting mechanisms for NTPP- and CTPP-containing proteins. One can speculate to the reason for the presence of such systems. The answer may be in response to events that occur earlier in the secretory pathway. Proteins upon entering the ER need to be folded properly to become competent for transport through the secretory pathway (Vitale et al., 1993). Properly folded soluble proteins lacking additional targeting information will be secreted from the cell by the default pathway (Dorel et al., 1989; Denecke et al., 1990). However, if a misfolded protein escapes the ER, instead of being secreted, the plant by developing a flexible recognition system can capture and deliver them to the vacuole for recycling. This type of salvage mechanism may be present in order to recycle and recover a loss of energy instead of secreting it to the extracellular space, thereby minimizing the loss. This is one explanation for the existence of a highly nonspecific CTPP recognition system. Interestingly, a poster presented by Loïc Faye (University of Rouen) at the 1995 Keystone meeting on Plant Cell Biology reported, that when the HDEL motif (ER retention signal) was added to the

C-terminus of a NTPP deletion mutant of sporamin (normally secreted), the protein escapes the ER and was found localized in the vacuole. However the plant needs a way to avoid this system and allow for specific proteins to be secreted. It has been shown that a glycan can mask vacuolar targeting determinants and highly charged C-terminal propeptides are not efficiently recognized by the sorting apparatus (Tague et al., 1990; Dombrowski et al., 1993). Therefore, additional research needs to be done to determine what properties a protein must possess to be competent for secretion from the cell or sorting to the vacuole.

Another area not well defined in plants is the transport and sorting of membrane proteins in the secretory pathway. It is still unclear if the default pathway for plant secretory membrane proteins is to the vacuole as in yeast, or to the plasma membrane as in mammalian system (See chapter 1). To date studies on plant vacuolar membrane transport has centered around the aquaporin homologue TIP, which has 6 membrane spanning domains (Gomez and Chrispeels, 1993). The presence of multiple transmembrane domains complicates the analysis of vacuolar membrane protein transport. Therefore, there is a need to identify a vacuolar membrane marker protein with a single transmembrane domain, in addition to resident Golgi membrane and plasma membrane localized proteins. The isolation and characterization of these proteins will provide a good model system for the study of membrane protein transport through the plant secretory pathway.

The study of yeast vacuolar protein sorting progressed at a slow pace until

the isolation of the *vps* mutants. As a result of their creation, there has been a rapid expansion in the knowledge of the field. The identification of genes and their products, combined with biochemical analyses have yielded valuable insights into the mechanisms involved in protein transport (for reviews see, Klionsky et al., 1990; Raymond et al., 1992). As a direct result of this genetic approach the identification of the carboxypeptidase Y receptor was achieved (Marcusson et al., 1994). Whereas, attempts to isolate the receptor by biochemical means had proved unsuccessful (Dr. Tom Stevens personal communication). In addition, the existence of an endosomal-like prevacuolar compartment in yeast was identified by the biochemical and immunocytochemical analyses of selected *vps* mutants (Vida et al., 1993). The question remains does such a compartment also exist in plants? If a similar compartment is present, then the delivery of proteins to the vacuole may require two vesicle budding and fusion events. This is a fundamental area of research which needs to be addressed for our basic understanding of the transport mechanism in plants.

Therefore, the creation of the vacuolar sorting mutants in *Arabidopsis* (described in Chapter 4), will provide the means to isolate relevant components of the sorting apparatus and provide a basic understanding of the mechanisms of protein transport. In addition, the study of these mutants may yield insights into other cellular processes, such as endocytosis, vacuolar biogenesis, cellular growth and protein turnover.

The isolation of conditional (temperature sensitive) mutants in vacuolar

sorting will greatly enhance the analysis of transport by biochemical means. One can envision that some mutants will block the transport of proteins at a particular point in the pathway. These mutants can then be utilized to isolate difficult to purify transport vesicles and endomembrane compartments, such as the TGN or prevacuolar compartment. Thus, by blocking a fusion step transport vesicles can be enriched. In addition, if the formation of vesicles at either the TGN or from a prevacuolar compartment is blocked, then there is a potential for the increased accumulation of transport intermediates causing the compartment to expand, which may simplify its isolation.

However, it will be the development of an *in vitro* vacuolar transport system that will provide the means by which the dissection of the transport process can be achieved. The *in vitro* system will allow for reconstitution experiments, providing a way for demonstrating the function of the protein, as well as means to study the requirements and cofactors necessary for transport.

Plants appear to have fundamental differences as compared to mammalian and yeast systems in the way soluble proteins are targeted to the vacuole. Therefore, the creation of vacuolar sorting mutants and the development of an *in vitro* vacuolar transport system assay will greatly enhance the feasibility to elucidate the mechanisms of the vacuolar protein sorting in plants.

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