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Determination of the Functional Elements within the Vacuolar Targeting Signal of Barley Lectin

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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 are 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, require 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 is 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 Bednarek and Raikhel, 1992; Chrispeels 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 sorting information for the vacuolar proteins phytohemagglutinin, 11S legumin, and patatin has been shown to be contained within portions of the mature proteins (for a review, see Bednarek and Raikhel, 1992). However, these protein regions share no sequence

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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 (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 1A. The CTPP is a hydrophobic 15–amino acid peptide that contains two acidic residues (Figure 1B) 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.

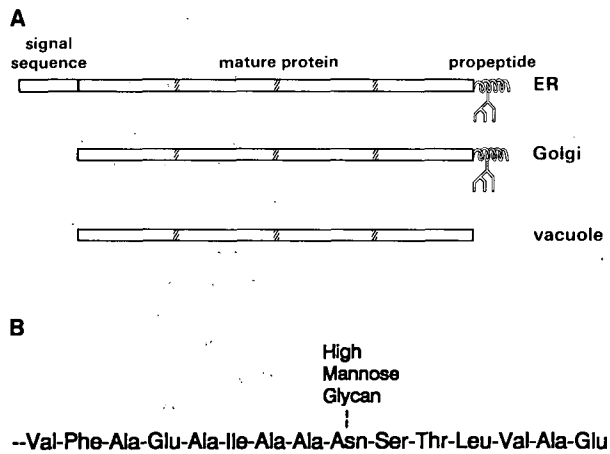

Figure 1. Schematic Representation of BL Maturation in the Secretory Pathway and the Carboxyl-Terminal Propeptide Amino Acid Sequence.

(A) The preproprotein of BL consists of a cotranslationally removed 26–amino acid signal sequence, four homologous domains of 43 amino acids of the mature protein, and a 15–amino acid carboxyl-terminal propeptide containing an N-linked high-mannose glycan. 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.

(B) 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.

	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	
	ADDITIONAL MUTATIONAL STUDIES	
32.*	CDG - VFAEPIPANSTLVAE	
33.*	CDG - VFAQAIAANSTLVAQ	
34.	CDG - VFAKAIAANSTLVAK	

Figure 2. Description and Summary of the Intracellular and Extracellular Distribution of BL-CTPP Mutants.

Each BL-mutant CTPP construct is represented by its 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 in which the C residue is involved in the formation of an 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 at equal to or greater than 95%, except construct 27, which is equal to or greater than 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.

RESULTS

Construction of the Mutant CTPPs of BL

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 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). 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 immunocytochemistry localized BL deletion mutant 3 (VFAEAI) to the vacuole, as shown in Figure 3A. Electron microscopic immunocytochemical analysis of transgenic plants expressing mutants 1 (VNSTLVAE) and 2 (VFAEAIAA) also showed specific localization to the 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 3C, the electron microscopic immunocytochemical analysis of tobacco plants expressing BL deletion mutant 10 showed no detectable labeling in the

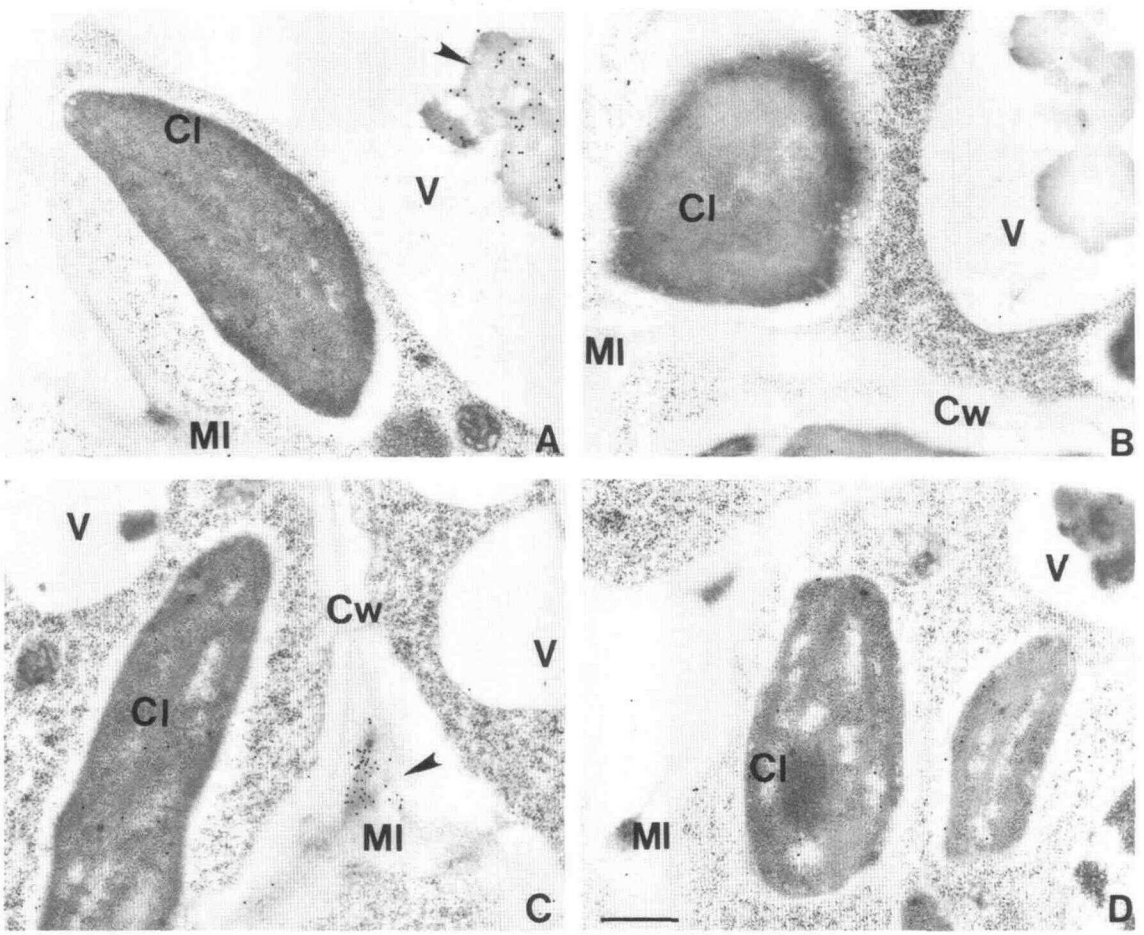

Figure 3. Immunocytochemical Localization of BL-Mutant CTPP Constructs 3 and 10.

(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 (arrows) 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; Ml, middle lamella; Cl, chloroplast.

vacuoles but was localized to the middle lamella of tobacco leaf cells. No specific labeling was detected in parallel experiments using nonimmune serum (Figures 3B and 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 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 because they contained equal to or less than 10% NADH–cytochrome *c* reductase relative to total protoplast-associated activity.

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 was analyzed using both transient and transgenic systems to ensure 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 to 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 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 radiolabeled BL was observed in the incubation medium, whereas the accumulation of BL intracellularly increased steadily over the same time period (data not shown).

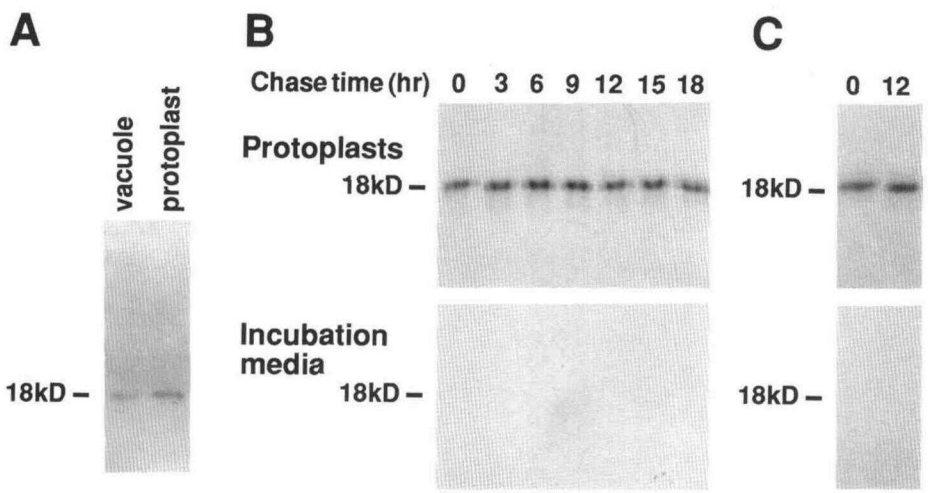

Figure 4. Subcellular Localization and Pulse-Chase Labeling Experiments of Transgenic and Transient Protoplasts Expressing BL-Mutant CTPP Construct 3.

(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.

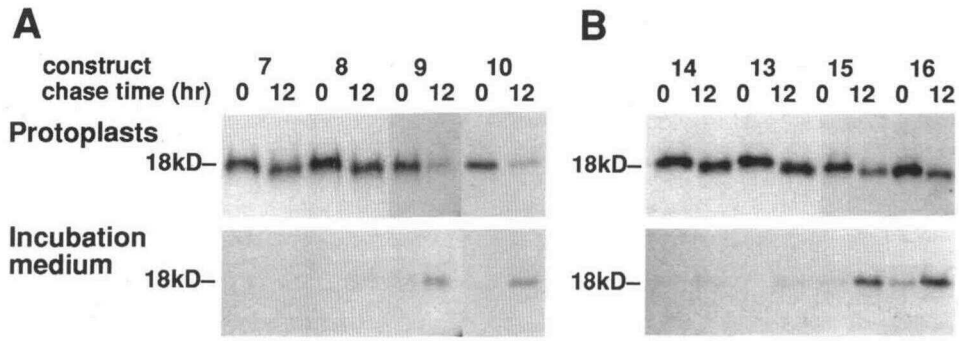

Figure 5. Deletion and Glycine Replacement Analysis.

(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.

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 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 mutants 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 four–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 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 5A, however, BL was secreted into the incubation media 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 3C, the electron microscopic 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 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).

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 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 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 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).

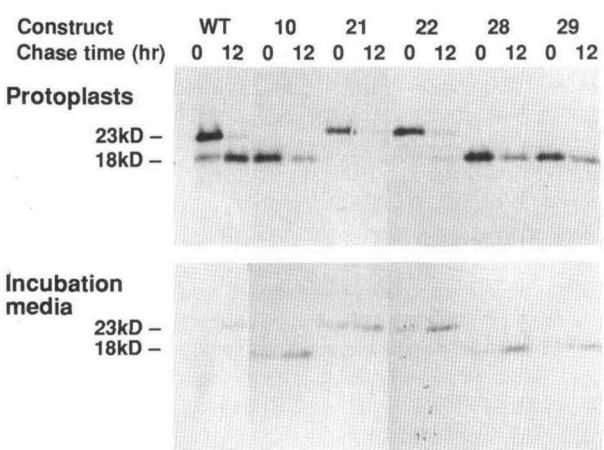

Figure 6. Disruption of Proper Sorting of BL by Carboxyl-Terminal Tandem Glycine Residues, Glycosylation Site Shift, and Artificial Propeptides.

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.

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 5B). Therefore, mutant 21 (VFAEAIAANSTLVAEGG) was constructed to analyze whether 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 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 inaccessible to the sorting apparatus in mutants 15 to 17 and 21. To confirm this hypothesis, we designed mutant 22 (VFAEAIAAGSTLV NATE) in which the site of glycan addition was shifted close to the carboxy terminus of the propeptide. As shown in Figure 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 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 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). BL-mutant CTPP constructs 23 to 27, 30, and 31 were retained intracellularly after pulse-chase labeling (data summarized in Figure 2). However, construct 27 (KDAEAD) did show a very low level of secretion (5 to 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 6).

Additional BL CTPP Mutants Addressing Structure

Mutant 32 (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 32 in transgenic plants indicated that the predicted amphipathic α-helix did not appear to have any significant effect on the CTPP processing or on the proper sorting of BL to the vacuole. BL-mutant CTPP construct 33 (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 34 (VFAKAIAANSTLVAK) exchanged basic lysine residues for the glutamic acid residues. Constructs 33 and 34 did not affect the proper processing or sorting of BL to the vacuole; however, construct 34 was not glycosylated (data not shown).

DISCUSSION

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 32 (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 indicates 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). 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 cannot 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 led 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. Based 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 three amino acids are required for proper sorting to the vacuole. A comparison of the last three amino acids of

the extracellular BL-mutant CTPP constructs in Figure 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 the 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 three–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 Bednarek and Raikhel, 1992; Chrispeels 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 (Subramani, 1992; van den Bosch et al., 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 that 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). 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.

METHODS

All standard recombinant DNA procedures used in this study were performed as described by 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 by Bednarek et al. (1990). BL-mutant CTPP clones were constructed either by modification of the CTPP coding region of the wild-type clone described by 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 by Bednarek et al. (1990). All carboxyl-terminal propeptide mutants of BL were identified and selected by ^{35}S-dideoxy sequencing of single-stranded DNA. The BL-mutant CTPP cDNAs were excised from pUC118 with XbaI (New England BioLabs). BL-mutant CTPP constructs (Figure 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, 32, and 33, as shown in Figure 2, and analyzed as described by Wilkins et al. (1990). Axenic shoot cultures of transformed tobacco were maintained and propagated by node cuttings on solid Murashige 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 by 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 by 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 milliliter. 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 $CaCl_2 \cdot 2H_2O$, 5 mM KCl, 5 mM glucose, pH 5.7). Viable protoplasts were visualized by fluorescein diacetate staining (Widholm, 1972), and the yields were quantitated using a hemocytometer counting chamber. Protoplasts were collected by centrifugation at 50*g* for 10 min, washed with 30 mL of BaMg solution (0.6 M betaine 15 mM $MgCl_2$, 3 mM 2-[*N*-morpholino]ethanesulfonic acid [Mes]-KOH, pH 5.7), and resuspended to a final concentration of 1.7×10^6 viable protoplasts per milliliter with the BaMg solution. Prior to adding plasmid DNA, 5×10^5 protoplasts were aliquoted to 15-mL polypropylene tubes (300 μL 1.7×10^6 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 50*g* 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 5.0×10^5 protoplasts per milliliter and transferred to 12-well tissue culture plates (Costar, Cambridge, MA.).

To examine expression of the BL 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 to 1100 Ci/mmol; ^{35}S-Met/Cys). If a pulse-chase analysis was performed after 8 hr of labeling, 100 μL of chase mix (100 mM 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 50*g* 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 16,000*g* 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 of 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.) microaffinity columns (Mansfield et al., 1988). After extensive washing of the column with TA buffer (50 mM Tris-acetate, pH 5.0, and 100 mM NaCl), BL was eluted with 150 μL of 200 mM *N*-acetylglucosamine and lyophilized. The inhibition of glycosylation by tunicamycin was performed as described by Bednarek and Raikhel (1991). The radiolabeled BL was analyzed by SDS-PAGE through 12.5% or 15% polyacrylamide gels and visualized by fluorography as detailed by Mansfield et al. (1988).

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REFERENCES

An, G., Ebert, P.R., Mitra, A., and Ha, S.B. (1988). Binary vectors. Plant Mol. Biol. Man. **A3,** 1–19.

Bednarek, S.Y., and Raikhel, N.V. (1991). The barley lectin carboxyl-terminal propeptide is a vacuolar protein sorting determinant in plants. Plant Cell **3,** 1195–1206.

Bednarek, S.Y., and Raikhel, N.V. (1992). Intracellular trafficking of secretory proteins. Plant Mol. Biol. **20,** 133–150.

Bednarek, S.Y., Wilkins, T.A., Dombrowski, J.E., and Raikhel, N.V. (1990). A carboxyl-terminal propeptide is necessary for proper sorting of barley lectin to vacuoles of tobacco. Plant Cell **2,** 1145–1155.

Chrispeels, M.J. (1991). Sorting of proteins in the secretory system. Annu. Rev. Plant Physiol. Plant Mol. Biol. **42,** 21–53.

Chrispeels, M.J., and Raikhel, N.V. (1992). Short peptide domains target proteins to plant vacuoles. Cell **68,** 613–616.

Flynn, G.C., Pohl, J., Flocco, M.T., and Rothman, J.E. (1991). Peptide-binding specificity of the molecular chaperone BiP. Nature **353,** 727–730.

Gould, S.J., Keller, G., Hosken, N., Wilkinson, J., and Subramani, S. (1989). A conserved tripeptide sorts proteins to peroxisomes. J. Cell Biol. **108,** 1657–1664.

Höfte, H., and Chrispeels, M.J. (1992). Protein sorting to the vacuolar membrane. Plant Cell **4,** 995–1004.

Holwerda, B.C., Padgett, H.S., and Rogers, J.C. (1992). Proaleurain vacuolar targeting is mediated by short contiguous peptide interactions. Plant Cell **4,** 307–318.

Johnson, L.M., Bankaitis, V.A., and Emr, S.D. (1987). Distinct sequence determinants direct intracellular sorting and modification of yeast vacuolar protease. Cell **48,** 875–885.

Klionsky, D.J., Banta, L.M., and Emr, S.D. (1988). Intracellular sorting and processing of a yeast vacuolar hydrolase: Proteinase A propeptide contains vacuolar targeting information. Mol. Cell. Biol. **8,** 2105–2116.

Kornfeld, S., and Mellman, I. (1989). The biogenesis of lysosomes. Annu. Rev. Cell Biol. **5,** 483–525.

Mansfield, M.A., Peumans, W.J., and Raikhel, N.V. (1988). Wheat-germ agglutinin is synthesized as a glycosylated precursor. Planta **173,** 482–489.

Matsuoka, K., and Nakamura, K. (1991). Propeptide of a precursor to a plant vacuolar protein required for vacuolar targeting. Proc. Natl. Acad. Sci. USA **88,** 834–838.

Miura, S., Kasuya-Arai, I., Mori, H., Miyazawa, S., Osumi, T., Hashimoto, T., and Fujiki, Y. (1992). Carboxyl-terminal consensus Ser-Lys-Leu-related tripeptide of peroxisomal proteins functions *in vitro* as a minimal peroxisome-targeting signal. J. Biol. Chem. **267,** 14405–14411.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. **15,** 473–497.

Nakamura, K., and Matsuoka, K. (1993). Protein targeting to the vacuole in plant cells. Plant Physiol. **101,** 1–5.

Neuhaus, J.-M., Sticher, L., Meins, F., Jr., and Boller, T. (1991). A short C-terminal sequence is necessary and sufficient for the targeting of chitinases to the plant vacuole. Proc. Natl. Acad. Sci. USA **88,** 10362–10366.

Pryer, N.K., Wuestehube, L.J., and Schekman, R. (1992). Vesicle-mediated protein sorting. Annu. Rev. Biochem. **61,** 471–516.

Raikhel, N.V., and Lerner, D.R. (1991). Expression and regulation of lectin genes in cereals and rice. Dev. Genet. **12,** 255–260.

Raikhel, N.V., Mishkind, M.L., and Palevitz, B.A. (1984). Characterization of a wheat germ agglutinin-like lectin from adult wheat plants. Planta **162,** 55–61.

Saalbach, G., Jung, R., Kunze, G., Saalbach, I., Adler, K., and Muntz, K. (1991). Different legumin protein domains act as vacuolar targeting signals. Plant Cell **3,** 695–708.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, 2nd ed. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).

Schroeder, R.M., Borkhsenious, O.N., Matsuoka, K., Nakamura, K., and Raikhel, N.V. (1993). Colocalization of barley lectin and sporamin in vacuoles of transgenic tobacco plants. Plant Physiol. **101,** 451–458.

Struhl, K. (1985). A rapid method for creating recombinant DNA molecules. Biotechniques **3,** 452–453.

Subramani, S. (1992). Targeting of proteins into the peroxisomal matrix. J. Memb. Biol. **125,** 99–106.

Valls, L.A., Hunter, C.P., Rothman, J.H., and Stevens, T.H. (1987). Protein sorting in yeast: The localization determinant of yeast vacuolar carboxypeptidase Y resides in the propeptide. Cell **48,** 887–897.

Valls, L.A., Winther, J.R., and Stevens, T.H. (1990). Yeast carboxypeptidase Y vacuolar targeting signal is defined by four propeptide amino acids. J. Cell Biol. **111,** 361–368.

van den Bosch, H., Schutgens, R.B.H., Wanders, R.J.A., and Tager, J.M. (1992). Biochemistry of peroxisomes. Annu. Rev. Biochem. **61,** 157–197.

Verner, K., and Schatz, G. (1988). Protein translocation across membranes. Science **241,** 1307–1313.

von Heijne, G. (1988). Transcending the impenetrable: How proteins come to terms with membranes. Biochim. Biophys. Acta **947,** 307–333.

Widholm, J.M. (1972). The use of fluorescein diacetate and phenosafranin for determining viability of cultured plant cells. Stain Technol. **47,** 189–194.

Wilkins, T.A., Bednarek, S.Y., and Raikhel, N.V. (1990). Role of propeptide glycan in post-translational processing and transport of barley lectin to vacuoles in transgenic tobacco. Plant Cell **2,** 301–313.

Wright, C.S. (1987). Refinement of the crystal structure of wheat germ agglutinin isolectin 2 at 1–8 Å resolution. J. Mol. Biol. **194,** 501–529.