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Mechanisms of Protein Sorting to the Plant Vacuole

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Sebastian York Bednarek

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Natasha Raikhel
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MECHANISMS OF PROTEIN SORTING TO THE PLANT VACUOLE

By

Sebastian York Bednarek

A DISSERTATION

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for the degree of

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Department of Botany and Plant Pathology

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ABSTRACT

MECHANISMS OF PROTEIN SORTING TO THE PLANT VACUOLE

By

Sebastian York Bednarek

In eukaryotic cells, proteins which enter the secretory system are either secreted or localized within one of the distinct compartments of the endomembrane system such as, the endoplasmic reticulum, the Golgi apparatus, or the vacuole/lysosome. Secretory proteins are sorted or retained within the endomembrane system by mechanisms which require specific targeting information contained within the structure of the protein, whereas proteins lacking such information follow a default pathway and are secreted from the cell. To understand the mechanisms of protein targeting to the plant vacuole we have defined the sorting signal of the vacuolar protein, barley lectin. The barley lectin proprotein contains a transiently associated glycosylated carboxyl-terminal propeptide which is removed prior to or concomitant with deposition of the mature protein in the vacuole. Expression of a cDNA encoding barley lectin in transgenic tobacco, results in the correct processing, maturation and accumulation of barley lectin in the vacuole. Similarly, barley lectin was localized in the vacuoles of transgenic plants expressing a mutated form of barley lectin in which the propeptide glycosylation site had been eliminated. Therefore, the carboxyl-terminal propeptide high-mannose glycan does not function as a vacuolar protein sorting determinant. Deletion of the carboxyl-terminal propeptide however, results in secretion of barley lectin. Furthermore, a

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fusion protein containing the extracellular cucumber chitinase and the barley lectin carboxyl-terminal propeptide is redirected to the vacuole of transgenic tobacco cells. These results demonstrate that the barley lectin carboxyl-terminal propeptide contains the sorting information necessary and sufficient for protein targeting to the plant vacuole.

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ACKNOWLEDGMENTS

In explaining what I do for "a living", Theresa explained to a friend after watching an immunoblot being developed, "He transfers liquid from one tube to another, mixes it, then transfers it to another tube and gets all excited when he sees purple lines on this special type of paper". In my opinion this described the process of science to the "T" (pardon the pun). Science does involve plenty of grunge work, and the product of this work can be either frustrating or incredibly exciting. I would like to express my sincere appreciation to Natasha for having made the process exciting. She gave me the freedom to think and to do the experiments I wanted to do. Her support, advice and extreme patience during were invaluable. I would also like to thank the members of my guidance committee, John Wilson, Pam Green and Hans Kende for their helpful comments and critical reviews. Special thanks are also due to Diccon Fiore, Jim Sellmer, and Tim Strabala and many other friends and coworkers for their continual support and/or friendly (often times well deserved) abuse. Thanks to Elwood for his meows and to Ryland for his smiles. I would like to thank my parents for their support, and for instilling in me the drive to succeed. Finally, it is to Theresa to whom I owe my greatest appreciation for her constant love, and encouragement.

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Plant cells like other eukaryotic cells, have compartmentalized many of their metabolic processes into discrete organelles. The processes whereby these organelles are formed and maintained is one of the central issues in cell biology and has been the focus of extensive research over the last two decades. With the exception of a few proteins made within the chloroplasts and mitochondria, the proteins present within the various subcellular organelles are all synthesized in the cytosol or on membrane bound ribosomes. The transport and sorting of proteins to their appropriate destinations is dependent on specific targeting signals present in the sequence or structure of the nascent protein.

The endomembrane system/secretory pathway consists of a series of organelles and transitional transport vesicles which mediate communication between these multiple compartments. Entry of proteins into the secretory pathway is dependent on a hydrophobic signal peptide typically found on the amino-terminus of most secretory proteins (von Heijne, 1988), which directs the protein into the endoplasmic reticulum (ER). From there, the flow of secretory proteins is directed through the organellar system, toward the cell surface. Resident proteins of the ER, Golgi and other endomembrane compartments such as the vacuole must however be actively retained or diverted from the "bulk flow" by additional targeting information (for a review see Chrispeels, 1991).

We are interested in understanding the mechanisms regulating the vacuolar sorting of the Gramineae lectins. Examination of the crystal structure of wheat germ agglutinin, a structural homologue of barley lectin, does not reveal any

regions that extend from the surface of the very compact mature protein (Wright, 1987) which might interact with a putative sorting receptor. Therefore, we hypothesized that information for vacuolar sorting is contained in the glycosylated carboxyl-terminal propeptides (CTPP) found on the Gramineae lectin proproteins.

This dissertation examines the sorting of barley lectin in transgenic tobacco and describes the identification of the probarley lectin CTPP as a vacuolar protein sorting signal.

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CHAPTER 2*

Role of propeptide glycan in posttranslational processing and transport of barley lectin to vacuoles in transgenic tobacco

* Originally published in *The Plant Cell*

Reference: Wilkins[1], T.A., Bednarek[1], S.Y. and Raikhel, N.V. (1990) *Plant Cell* **2**, 301-313

[1] Both authors contributed equally to this work

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ABSTRACT

Mature barley lectin is a dimeric protein comprised of two identical 18 kd polypeptides. The subunits of barley lectin are initially synthesized as glycosylated proproteins which are post-translationally processed to the mature protein preceding or commensurate with deposition of barley lectin in vacuoles. To investigate the functional role of the glycan in processing and intracellular transport of barley lectin to vacuoles, the sole N-linked glycosylation site residing within the COOH-terminal propeptide of barley lectin was altered by site-directed mutagenesis. cDNA clones encoding wild-type (*wt*) or glycosylation-minus (*gly*) barley lectin preproproteins were placed under the transcriptional control of the cauliflower mosaic virus 35S promoter and introduced into *Nicotiana tabacum* cv. Wisconsin 38. Barley lectin synthesized from both the *wt* or *gly* constructs was processed and correctly targeted to vacuoles of tobacco leaves. Localization of barley lectin in vacuoles processed from the nonglycosylated *gly* proprotein indicated that the high mannose glycan of the barley lectin proprotein was not essential for targeting barley lectin to vacuoles. However, pulse-chase labeling experiments demonstrated that the glycosylated *wt* proprotein and the nonglycosylated *gly* proprotein were differentially processed to the mature protein and transported from the Golgi complex at different rates. These results implicate an indirect functional role for the glycan in post-translational processing and transport of barley lectin to vacuoles.

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INTRODUCTION

Many proteins entering the endomembrane system of the secretory pathway are modified in the lumen of the rough endoplasmic reticulum (RER) by the covalent attachment of high-mannose oligosaccharide sidechains (glycans) to selective asparagine (N) residues. The N-linked high-mannose glycans may be subsequently modified to complex glycans as the glycoprotein traverses through the Golgi complex. Inhibition of glycosylation by site-directed mutagenesis or the drug tunicamycin apparently does not affect the synthesis, intracellular transport, or function of some glycoproteins (reviewed in Olden *et al.*, 1985). However, the N-linked glycans of other glycoproteins have been shown to influence protein folding (Machamer and Rose, 1988; Matzuk and Boime, 1988), oligomerization (Matzuk and Boime, 1988), stability (reviewed in Olden *et al.*, 1985), and protein targeting (Kornfeld, 1987).

Studies exploring the functional role of N-linked oligosaccharides in plants are limited. Proteins modified by N-linked glycosylation may be localized within a subcellular compartment or in the cell wall. The glycans of the vacuolar protein phytohemagglutinin (PHA) and the secreted α-amylase of rice, however, are not required for transport and targeting of these proteins to their respective compartments (Bollini, *et al.*, 1985; Voelker, *et al.*, 1989; Akazawa and Hara-Nishimura, 1985). In fact, many vacuolar and secretory proteins are not glycoproteins, suggesting that N-linked oligosaccharide side-chains do not generally function as sorting signals. A functional role for the glycan of the

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vacuolar protein concanavalin A, however, is implicated in the intracellular processing and transport of this protein (Faye and Chrispeels, 1987). Mature ConA is not a glycoprotein, although it is synthesized as a glycosylated precursor (proConA) (Herman, *et al.*, 1985). The mature ConA polypeptide is generated by the excision of an internal glycopeptide from proConA and subsequent ligation of the two resultant polypeptides (Bowles *et al.*, 1986). Inhibition of N-linked glycosylation with the inhibitor tunicamycin significantly impedes transport of proConA from the ER/Golgi compartment to vacuoles (Faye and Chrispeels, 1987).

The post-translational processing of Gramineae lectins, which are soluble vacuolar proteins, is distinctive from PHA and ConA. The mature lectins of wheat, barley, and rice are 36 kd dimers assembled from two identical 18 kd subunits (Rice and Etzler, 1974; Peumans, *et al.*, 1982a; Peumans, *et al.*, 1983). Similar to ConA, mature lectins are not glycoproteins. However, the lectin subunits are initially synthesized as glycosylated proproteins in wheat (Raikhel and Wilkins, 1987; Mansfield, *et al.*, 1988), barley (Lerner and Raikhel, 1989) and rice (Wilkins and Raikhel, unpublished results). The sole N-linked glycosylation site (Asn-X-Ser/Thr) resides within the propeptide located at the COOH-terminal of these proproteins. Endo-β-N-acetylglucosaminidase H (Endo H) studies demonstrate that the oligosaccharide side-chain of these proproteins is a high mannose glycan with a molecular weight of approximately 2 kd (Lerner and Raikhel, 1989; Smith and Raikhel, 1989; Wilkins and Raikhel, unpublished results). The COOH-terminal N-glycopeptide of the proprotein is post-translationally removed prior to or concomitant with deposition of the mature protein in vacuoles. The transient

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glycosylation of the Gramineae lectin proproteins provides a unique opportunity to investigate the molecular mechanisms which mediate the maturation and targeting of mature lectins to vacuoles. In this study, we have examined the synthesis, assembly, processing, and subcellular localization of barley lectin in transgenic tobacco. In addition, the functional role of the barley lectin propeptide glycan was assessed by introducing a mutant barley lectin cDNA into tobacco. The N-linked glycosylation site within the COOH-terminal propeptide in the mutant barley lectin cDNA was modified by site-directed mutagenesis to prevent the co-translational N-glycosylation of the barley lectin proprotein. The results established that both the wild-type and mutant barley lectin are expressed, correctly processed, and transported to vacuoles of tobacco leaves. However, the rates of post-translational processing through the RER/Golgi complex were distinctive for the wild-type or mutant barley lectin proproteins.

RESULTS

Inactivation of N-linked glycosylation site of barley lectin proprotein by site-directed mutagenesis

The cDNA clone pBLc3 (Lerner and Raikhel, 1989) encodes the 23 kd preproprotein of barley lectin. The preproprotein is comprised of a 2.5 kd signal sequence, the 18 kd mature protein, and a 1.5 kd COOH-terminal propeptide (see Figure 1B). In barley embryos, the proprotein is modified by the addition of a 2 kd high-mannose oligosaccharide sidechain to the sole N-linked glycosylation site

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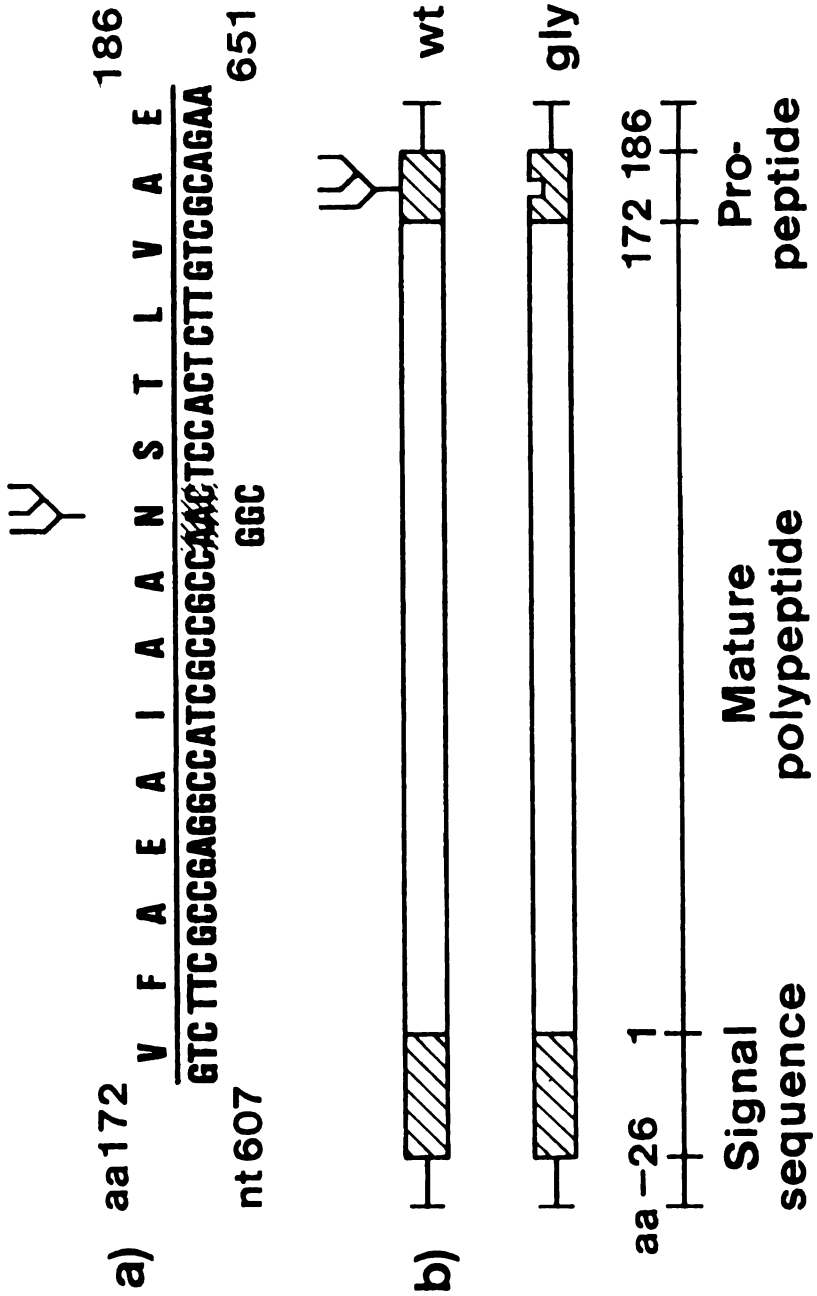
located within the COOH-terminal propeptide at Asn_{180}-Ser-Thr_{182} (Figure 1A). To further investigate the assembly, post-translational processing and transport of barley lectin to vacuoles, the cDNA encoding barley lectin was introduced into tobacco and the post-translational processing of monocot barley lectin examined in this heterologous dicot system. The barley lectin cDNA was subcloned into the binary plant expression vector pGA643 (An, *et al.*, 1988) under the transcriptional control of the cauliflower mosaic virus (CaMV) 35S promoter. *Agrobacteria*-mediated transformation of tobacco (*Nicotiana tabacum* cv. Wisconsin 38) was accomplished via the leaf disc method of Horsch, *et al.* (1988). Both the constructs and kanamycin-resistant tobacco transformants containing the barley lectin cDNA were designated by the code *wt* (Figure 1B).

The glycosylated COOH-terminal propeptide is transiently associated with barley lectin proprotein but not with the nonglycosylated mature protein localized in vacuoles. Mature barley lectin is generated by the cleavage of the N-linked glycosylated propeptide from the proprotein preceding or concomitant with the deposition of barley lectin in vacuoles. To assess the functional role of the N-linked high mannose glycan in the assembly, processing, and targeting of barley lectin to vacuoles, site-directed mutagenesis was performed to alter the N-linked glycosylation site within the COOH-terminal propeptide. The N-linked glycosylation site was altered by converting Asn_{180} (AAC) to a Gly_{180} (GGC) residue using a 16-base mutagenic synthetic oligonucleotide spanning the glycosylation site at Asn_{180}-Ser-Thr_{182} (Figure 1A). The mutant barley lectin cDNA was subcloned into pGA643 and transformed into tobacco. Constructs and kanamycin-resistant tobacco plants

Figure 1. Alteration of the N-linked glycosylation site of barley lectin by site-directed mutagenesis and organization of the wild-type (*wt*) and mutant (*gly*) barley lectin cDNAs introduced into tobacco.

(**A**) The 15 amino acid COOH-terminal propeptide of barley lectin (aa 172 through aa 186) and the corresponding nucleotide sequence (nt 607 to 651). The N-linked glycosylation site (Asn_{180}-Ser-Thr_{182}) is depicted by attachment of a high mannose glycan tree to Asn (N) residue 180. The N-linked glycosylation site at N_{180} (shaded codon) was converted to a Gly residue (GGC) by site-directed mutagenesis to generate a barley lectin mutant which cannot be glycosylated. (**B**) The structure of *wt* and *gly* barley lectin cDNA clones subcloned into the plant expression vector pGA643 (An et al., 1988).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containing the mutant barley lectin were designated as *gly* (Figure 1B).

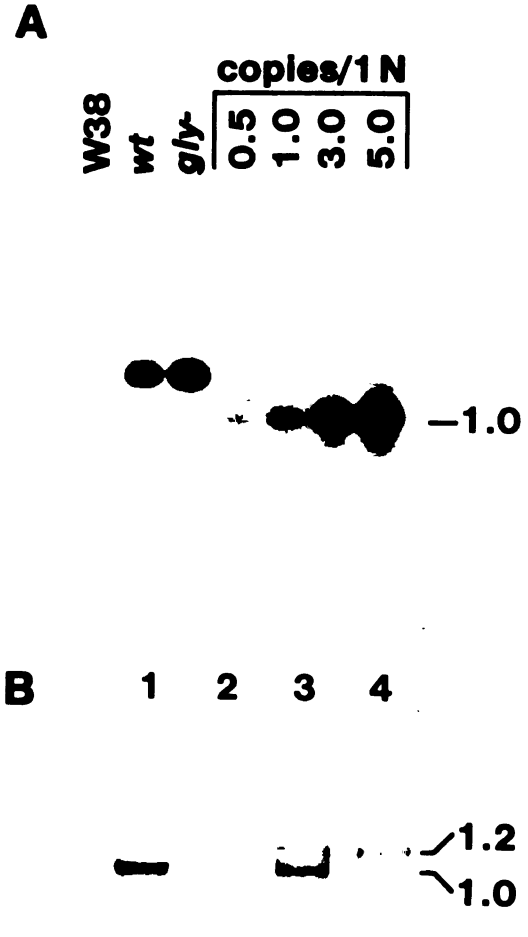
Detection of barley lectin cDNA and mRNA in transgenic tobacco

The structure and stable integration of *wt* and *gly* barley lectin cDNA into the tobacco genome was examined in independent transformants by Southern blot analysis. A radiolabeled restriction fragment containing a portion of the barley lectin cDNA and the T-DNA left border of pGA643 was used to probe tobacco genomic DNA restricted with HindIII. Three HindIII-restriction fragments (5 kbp to 9.0 kbp) and five fragments (18 kbp and 2.8 kbp to 4.0 kbp) were detected in tobacco genomic DNA isolated from *wt* and *gly* transformants, respectively (data not shown). Gene reconstruction experiments (Figure 2A) were performed with EcoRI-restricted tobacco DNA and purified BLc3 insert titered at 0.5-, 1.0-, 3.0-, and 5-copy equivalents per tobacco genome. Hybridization of gene reconstruction experiments with radiolabeled BLc3 indicated the presence of 3-copies of *wt* and 5-copies of *gly* barley lectin cDNA integrated into the tobacco genome of the individual transformants presented in Figure 2A. No hybridization was observed between barley lectin and tobacco DNA in untransformed plants (W38, Figure 2A) or in transgenic plants containing only the vector pGA643 (data not shown).

The relative levels of mRNA encoding *wt* or *gly* barley lectin in transgenic tobacco was investigated by Northern blot analysis. The Northern blot in Figure 2B represents the accumulation of *wt* and *gly* barley lectin steady-state mRNA in total RNA isolated from transgenic tobacco leaves detected by ^{32}P-labeled barley lectin cDNA (BLc3). Two mRNA species of 1.2 kb and 1.0 kb were observed in

Figure 2. Gene reconstruction analysis and accumulation of steady-state RNA levels of barley lectin in transgenic tobacco.

(A) Southern blot containing 12 ug of tobacco genomic DNA restricted with EcoRI and probed with a radiolabeled HindIII-SalI restriction fragment from a pGA643 construct containing barley lectin cDNA. Reconstruction lanes represent 0.5-, 1.0-, 3.0-, and 5.0-gene copy equivalents of barley lectin pBLc3 cDNA insert (Lerner and Raikhel, 1989) per haploid genome of tobacco. Tobacco DNA was isolated from untransformed tobacco (cv. W38) and transgenic tobacco plants containing cDNAs encoding wild-type (*wt*) or mutant (*gly*) barley lectin preproproteins. Approximate size of fragments in kbp are positioned to the right. **(B)** Northern blot containing 25 ug of total RNA isolated from developing barley embryos **(lane 1)**, untransformed tobacco (cv. W38) **(lane 2)** and transgenic tobacco plants containing *wt* **(lane 3)** or *gly* **(lane 4)** barley lectin cDNA constructs. The size of barley lectin mRNA species is indicated in kb to the right of the blot.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tobacco transformants containing either the *wt* or *gly* barley lectin (Lanes 3 and 4, respectively, Figure 2B). The 1.0 kb barley lectin mRNA in tobacco transformants (Lanes 3 and 4, Figure 2B) corresponds in length to the 1.0 kb barley lectin mRNA in developing barley embryos (Lane 1, Figure 2B; Lerner and Raikhel, 1989). The 1.2 kb mRNA species was unique to transgenic tobacco plants and presumably represented utilization of an alternate polyadenylation site contained within the termination sequences of the plant expression vector pGA643 (An *et al.*, 1988). Examination of individual transformants revealed the differential accumulation of the 1.2 kb and 1.0 kb lectin mRNAs in both *wt* and *gly* plants (Lanes 3 and 4, Figure 2B; data not shown). However, densitometer scanning of the autoradiograph indicated that the overall accumulation of steady-state *wt* and *gly* barley lectin mRNAs were very similar. No hybridization was observed in total RNA isolated from transgenic plants containing only the vector pGA643 (data not shown) or in untransformed tobacco (Lane 2, Figure 2B) probed with barley lectin cDNA.

Expression and assembly of active barley lectin in tobacco

Gramineae lectins possess the ability to specifically bind oligomers of the carbohydrate N-acetylglucosamine (GlcNAc). Since the carbohydrate binding site of wheat germ agglutinin (WGA) is comprised of amino acids contributed by both monomeric subunits (Wright, 1980), the assembly of active WGA is therefore contingent upon the formation of the dimer. Barley lectin shares 95% amino acid

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homology with WGA, including conservation of amino acids involved in carbohydrate binding (Lerner and Raikhel, 1989). This conservation is exemplified by the ability to form active heterodimers *in vitro* from monomeric subunits of WGA and barley lectin (Peumans, *et al.*, 1982b). Hence, the mechanism of dimerization and carbohydrate binding of WGA and barley lectin are presumably identical.

In order to determine if barley lectin was synthesized and assembled into an active lectin in transgenic tobacco plants, crude protein extracts prepared from *wt* or *gly* tobacco transformants were fractionated on an immobilized GlcNAc affinity matrix. The affinity-purified fractions were separated by SDS-PAGE and analyzed by immunoblotting (Figure 3). Since barley lectin and WGA are antigenically indistinguishable (Stinissen, *et al.*, 1983), polyclonal anti-WGA antiserum was used to detect barley lectin on immunoblots. The 18 kd mature subunit of barley lectin was readily discernible in *wt* or *gly* transgenic tobacco leaves (Lanes 3 and 4, respectively, Figure 3). Detection of mature 18 kd polypeptides on immunoblots following affinity chromatography (Figure 3) indicated that barley lectin is synthesized and assembled as an active GlcNAc-binding lectin in both *wt* and *gly* tobacco transformants. Anti-WGA antiserum does not cross-react with any polypeptide in untransformed tobacco (Lane 2, Figure 3). Similar results were obtained on immunoblots prepared from roots of *wt* and *gly* transgenic tobacco plants (results not shown).

The accumulation of barley lectin in *wt* and *gly* tobacco plants was quantitated in total acid soluble protein extracts from transgenic tobacco leaves using double-bind ELISA. A range of 800 ng to 2 ug of affinity-purified barley

Figure 3. Immunoblot detection of mature barley lectin in *wt* and *gly* tobacco transformants.

Acid soluble protein extracts from *wt* (**lane 3**) and *gly* (**lane 4**) transformed and untransformed (**lane 2**) tobacco leaves were concentrated by ammonium sulfate precipitation. Barley lectin was affinity purified as described in Materials and Methods, separated on SDS-PAGE, and electroblotted onto nitrocellulose. Immunodetection of barley lectin was performed with polyclonal anti-WGA antiserum and protein A-conjugated alkaline phosphatase. **Lane 1** is a control lane containing 1 ug of purified WGA. The molecular mass of mature WGA and barley lectin subunits in kd is shown on the left.


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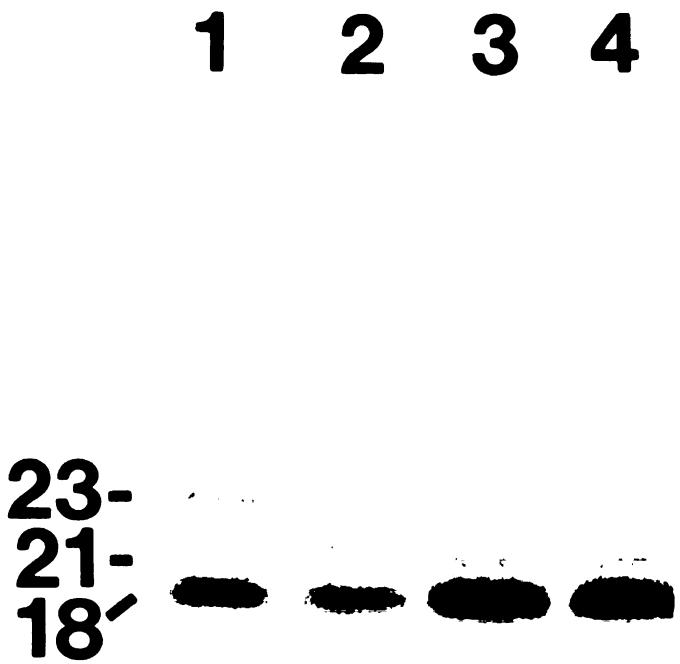
lectin per 1 g/fw leaf tissue was recovered from *wt* and *gly* tobacco transformants. The accumulation of barley lectin in tobacco leaves corresponded to 0.2% to 0.5% of total acid-soluble leaf proteins.

Synthesis of wild-type (wt) and mutant (gly⁻) barley lectin proproteins in tobacco protoplasts

In barley embryos, barley lectin is initially synthesized as a 23 kd glycosylated proprotein (Stinissen, *et al.*, 1985; Lerner and Raikhel, 1989). To ensure that barley lectin was synthesized and processed by similar mechanisms in tobacco, the post-translational modifications of radiolabeled barley lectin precursors in transgenic tobacco were examined. Tobacco protoplasts were prepared from axenic cultures and pulse-labeled for 12 hrs in the presence of ^{35}S-Trans label. Radiolabeled barley lectin was recovered from tobacco protoplasts by affinity chromatography on immobilized GlcNAc columns. Following affinity chromatography, eluant fractions were treated with Endo H, an enzyme which specifically cleaves high mannose oligosaccharide side-chains between the GlcNAc residues of the glycan core. Radiolabeled proteins incubated in the presence or absence of Endo H were analyzed following separation by SDS-PAGE and fluorography (Figure 4). In addition to the mature 18 kd subunit, a 23 kd polypeptide was also evident in pulse-labeled *wt* tobacco protoplasts (Lane 1, Figure 4). The majority of the 23 kd polypeptide was converted to a 21 kd protein following treatment with Endo H (Lane 2, Figure 4), indicating that the 23 kd polypeptide contained a 2 kd high mannose glycan. These results infer that the

Figure 4. Endo H digestion of radiolabeled barley lectin isolated from transgenic tobacco.

Radiolabeled barley lectin was affinity purified from *wt* (**lanes 1 and 2**) and *gly* (**lanes 3 and 4**) tobacco protoplasts pulse-labeled for 12 hr. Duplicate samples were incubated at 37°C for 23 hr in the absence (**lanes 1 and 3**) or presence (**lanes 2 and 4**) of Endo H. Samples were lyophilized and separated by SDS-PAGE. The position and molecular mass (kd) of barley lectin *wt* and *gly* proproteins (23 and 21 kd, respectively) and mature barley lectin (18 kd) are indicated to the left.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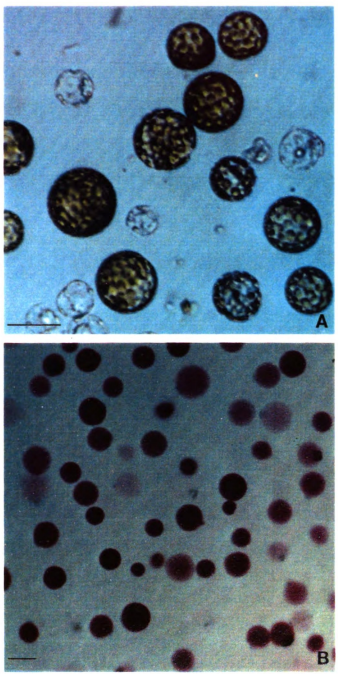
signal sequence had been cleaved and that the synthesis and processing of barley lectin precursors in tobacco was analogous to processing mechanisms in barley. As expected, the *gly* barley lectin was synthesized as a 21 kd proprotein (Lane 3, Figure 4) which was resistant to Endo H (Lane 4, Figure 4). Comparison of the *wt* and *gly* 21 kd polypeptides (Lanes 2 and 4, respectively, Figure 4) shows a slight disparity in migration of these two proproteins. The slower migration of the *wt* 21 kd polypeptide was due to the presence of a GlcNAc residue (M, 221.2), which remained attached to Asn_{180} of the propeptide after enzymatic deglycosylation with Endo H (Kobata, 1984). Thus, both the wt and gly barley lectins were synthesized as the predicted glycosylated 23 kd and nonglycosylated 21 kd proproteins, respectively, and processed to 18 kd mature polypeptides similarly in transgenic tobacco and barley.

Subcellular localization of wt and gly barley lectin in vacuoles

Barley lectin is localized in vacuoles in the peripheral cell-layers of embryonic and adult root caps of barley (Mishkind, *et al.*, 1983; Lerner and Raikhel, 1989). The subcellular location of *wt* and *gly* barley lectin in transgenic tobacco was ascertained by a combination of organelle fractionation, immunoblot analysis and EM immunocytochemistry. Protoplasts were prepared from both *wt* and *gly* transgenic tobacco plants (Figure 5A). Vacuoles were released from protoplasts (Figure 5B) and purified by centrifugation on a discontinuous ficoll gradient system. The purity of the vacuole preparation was evaluated by determining the

Figure 5. Isolation of vacuoles from tobacco protoplasts expressing *wt* or *gly* barley lectin.

(A) Protoplasts were prepared by enzymatic digestion of tobacco leaves collected from axenically cultured transgenic plants. Bar = 10 um. **(B)** Vacuoles stained with neutral red were isolated from tobacco protoplasts by centrifugation on a discontinuous 5%/10% Ficoll step gradient. Stained vacuoles were collected from the 0%/5% Ficoll interface and purified on a second 5%/10% Ficoll step gradient. Bar = 10 um.


A
B

enzymatic activity of two vacuolar-specific enzymes (acid phosphatase and α-mannosidase) and a peroxisomal enzyme (catalase) in vacuoles and protoplasts. Catalase was employed as an extravacuolar enzyme marker for two reasons. One, peroxisomes are very fragile and consequently lyse during preparation of vacuoles, thereby liberating catalase into the cell lysate. Secondly, the high specific activity of catalase was readily detectable at very low concentrations in cell lysates. As shown in Table 1, the relative enzymatic activity of the vacuolar enzyme markers in vacuoles isolated from *wt* or *gly* protoplasts approaches 100%. Less than 2% of catalase activity was associated with the vacuoles, indicating that the vacuoles (Figure 5B) were essentially free of contaminating cytosol and unbroken protoplasts.

Protoplast and vacuole fractions from *wt* and *gly* tobacco plants were examined for the presence of barley lectin by immunoblot analysis. Barley lectin was purified by affinity chromatography from a protein lysate representing an equivalent number of *wt* or *gly* protoplasts and vacuoles and analyzed on immunoblots (Figure 6) with polyclonal anti-WGA antiserum. The 18 kd mature subunit of barley lectin was readily discernible in protoplasts isolated from *wt* or *gly* tobacco plants (Lanes 2 and 4, Figure 6). Immunoblot analysis also revealed the presence of mature barley lectin in both *wt* and *gly* vacuoles (Lanes 3 and 5, Figure 6). These results indicated that barley lectin was correctly targeted to vacuoles in tobacco. Moreover, the absence of the propeptide glycan did not apparently preclude the targeting of barley lectin to tobacco vacuoles.

The vacuolar distribution of barley lectin in *wt* and *gly* transgenic tobacco leaves

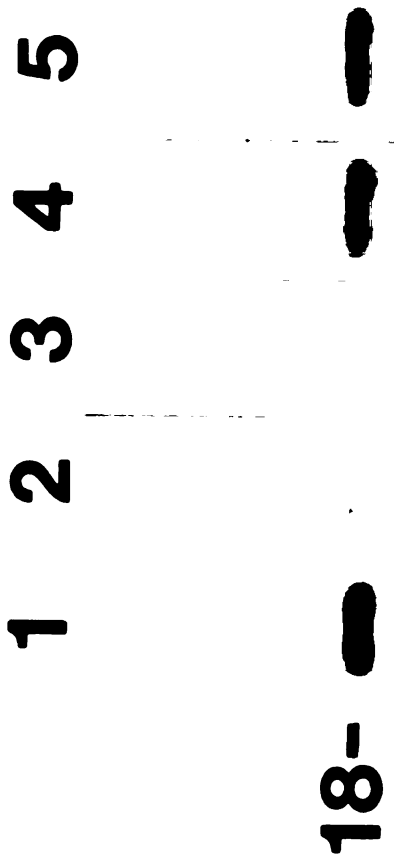
Table 1. Relative Enzyme Activity (%) in Vacuoles Prepared from Transgenic Tobacco Protoplasts

	wt	*gly*
Vacuole-specific enzymes		
α-mannosidase	106.8 ± 8.1	102.5 ± 1.5
acid phosphatase	85.6 ± 6.8	98.6 ± 10.3
Extravacuolar enzyme		
catalase	<2.0	<2.0

Enzyme activities of two vacuole-specific enzyme markers and an extravacuolar enzyme were determined in protoplast and vacuole fractions prepared from transgenic tobacco plants expressing *wt* or *gly* barley lectin. Enzyme activity in vacuoles is expressed as a percent of the activity determined in the same number of protoplasts. Results represent the mean ± SD calculated from three individual experiments.

Figure 6. Immunodetection of mature barley lectin in protoplasts and vacuoles isolated from *wt* and *gly* trangsenic tobacco plants.

Mature barley lectin detected in protoplasts and vacuoles prepared from a tobacco plant expressing *wt* barley lectin (**lanes 2 and 3**, respectively) or *gly* barley lectin (**lanes 3 and 5**, respectively). **Lane 1** is affinity purified mature WGA. The molecular mass of mature WGA and barley lectin is denoted on the left in kd.


5
4
3
2
1
18-

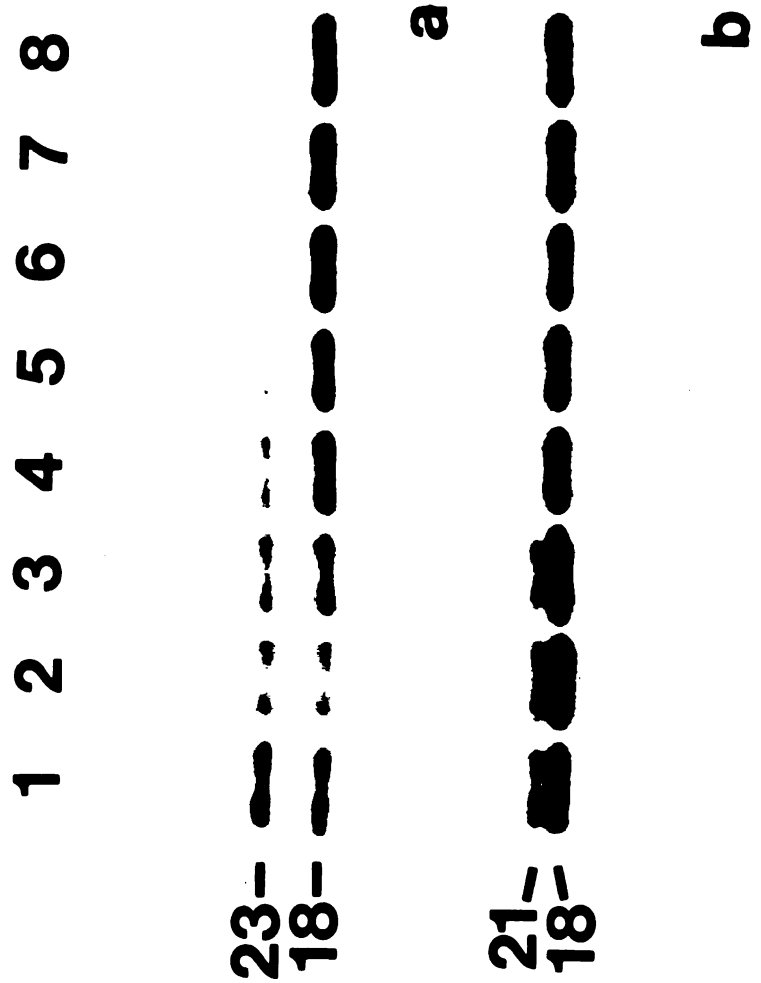
was also confirmed by EM immunocytochemistry (results not shown). No immunoreactive component was observed in the cytoplasm of transgenic tobacco plants (data not shown).

Kinetics of intracellular processing of wt and gly barley lectin in transgenic tobacco

Pulse-chase experiments were performed to assess the influence of the high-mannose glycan contained within the propeptide of the barley lectin proprotein on the rate of post-translational processing and accumulation of mature barley lectin in tobacco vacuoles. Both *wt* and *gly* tobacco protoplasts were pulse-labeled for 10 hr in the presence of ^{35}S-Trans label and chased with unlabeled methionine and cysteine for an additional 10 hrs. At specified intervals during the chase period, radiolabeled barley lectin was recovered from lysed protoplasts by affinity chromatography and analyzed by SDS-PAGE and fluorography, the results are shown in Figure 7. The 23 kd *wt* proprotein and the 21 kd gly· proprotein as well as the 18 kd mature barley lectin polypeptide were present in pulse-labeled protoplasts (Lane 1, Figure 7A and 7B). During the chase period, both the *wt* and *gly* radiolabeled proproteins gradually disappeared over time (Figure 7A and 7B, respectively). The disappearance of the barley lectin proproteins was accompanied by a corresponding increase in the level of the 18 kd mature protein. The radioactivity of the each band was quantitated by scanning densitometry. Conversion of both *wt* and *gly* proproteins to the mature polypeptide appeared to exhibit first order kinetics. Half-life ($t_{1/2}$) determinations of the *wt* or *gly* barley lectin proproteins indicated that the *gly* 21 kd proprotein ($t_{1/2}$ = 1.0 hr) are

Figure 7. Pulse-chase labeling experiments of tobacco protoplasts expressing *wt* or *gly* barley lectin.

Tobacco protoplasts expressing *wt* (**Panel A**) or *gly* (**Panel B**) barley lectin were pulse-labeled for 10 hr and chased for 0, 1, 2, 3, 4, 6, 8, and 10 hr (**lanes 1-8**). Radiolabeled barley lectin was affinity purified from lysed protoplasts and the eluants subjected to SDS-PAGE and fluorography. The position and molecular mass of the *wt* (23 kd) and *gly* (21 kd) barley lectin proproteins and the 18 kd mature polypeptide are denoted on the left.


1 2 3 4 5 6 7 8
23–
18–
a
21–
18–
b

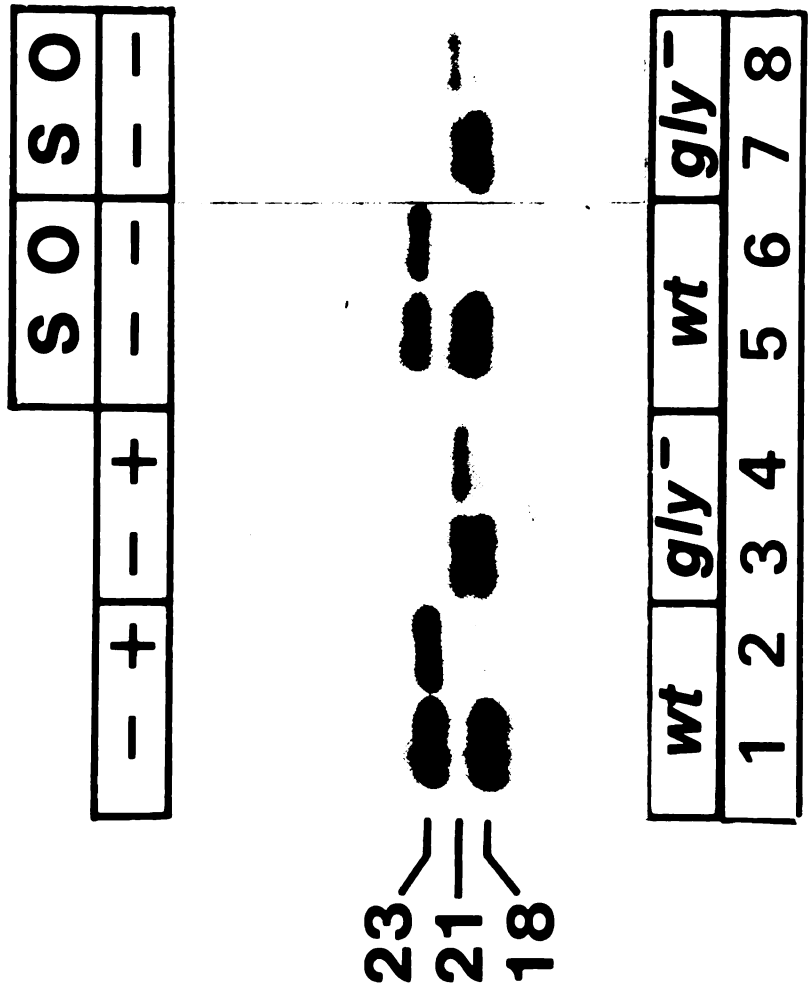
processed to the mature protein at least 2-fold faster than the *wt* 23 kd proprotein ($t_{1/2}$ = 2.0 hr). The disappearance of both *wt* and *gly* proproteins displayed linear first order kinetics in all experiments. Half-life estimates of *wt* and *gly* proprotein were compiled from three independent pulse-chase labeling experiments encompassing two individual transformants for each genotype. These results indicated that *wt* and *gly* barley lectin proproteins were differentially processed with distinctive rates during transport through the endomembrane system of the secretory pathway.

Post-translational processing of barley lectin in transgenic tobacco

Processing of the proprotein to mature barley lectin involves the selective removal of the COOH-terminal glycopeptide from the proprotein. To address the events involved in the post-translational processing of the proprotein of barley lectin, *wt* and *gly* tobacco protoplasts were pulse-labeled in the presence of the inhibitor monensin. Monensin is an ionophore that primarily disrupts transport vesicles and protein sorting from the *trans*-cisternae of the Golgi complex (Tartakoff, 1983; Chrispeels, 1983). Following a 1 hr preincubation in the presence of monensin, both *wt* and *gly* tobacco protoplasts were subsequently pulse-labeled for 12 hr. Radiolabeled barley lectin was affinity purified from lysed protoplasts and analyzed by SDS-PAGE and fluorography. The effect of monensin on the post-translational processing of *wt* and *gly* barley lectin proproteins in tobacco is presented in Figure 8. In the absence of monensin, both the 18 kd mature protein and the *wt*

Figure 8. Inhibition of proteolytic processing of barley lectin proproteins in the presence of monensin.

Tobacco protoplasts expressing *wt* or *gly* barley lectin were pulse-labeled for 12 hr in 0.1% ethanol (-) or 50 uM monensin, 0.1% ethanol (+). Radiolabeled barley lectin was affinity purified from a portion of the protoplasts and analyzed by SDS-PAGE and fluorography. Soluble (**S**) and organelle (**O**) fraction from the remaining protoplasts were separated by Sepharose-4B chromatography. Radiolabeled barley lectin in subcellular fractions of pulse-labeled tobacco protoplasts were affinity purified and fractionated by SDS-PAGE and treated for fluorography. The position and molecular mass (kd) of barley lectin proproteins and mature polypeptide are indicated to the left.


S O S O
- + - + - - - -
23
21
18
wt gly⁻ wt gly⁻
1 2 3 4 5 6 7 8

or *gly* proproteins were evident in pulse-labeled protoplasts (Lanes 1 and 3, respectively, Figure 8). However, the preponderance of barley lectin radiolabeled in the presence of monensin were the 23 kd *wt* or 21 kd *gly* proproteins (Lanes 2 and 4, respectively, Figure 8), indicating that monensin effectively inhibited processing of the proproteins to the mature polypeptide. Densitometer scanning of trace levels of 18 kd mature protein observed in both *wt* and *gly* protoplasts (Lanes 2 and 4, Figure 8) established that less than 4% of the proproteins were converted to the mature protein in the presence of monensin.

Monensin primarily disrupts intracellular vesicular transport and consequently results in extracellular secretion of lysosomal proteins (Tartakoff, 1983). Pea vicilin (Craig and Goodchild, 1984) and ConA (Bowles, *et al.*, 1986) accumulate at the cell surface and in the periplasmic space between the cell wall and the plasma membrane in cotyledons treated with monensin. Thus, the presence and relative abundance of radiolabeled barley lectin was examined in the culture media of pulse-labeled *wt* and *gly* tobacco protoplasts incubated in the presence or absence of monensin. Radiolabeled barley lectin was isolated from the culture media by affinity chromatography and subsequently analyzed by SDS-PAGE and fluorography. Radiolabeled barley lectin was not discernible in the culture media of either *wt* or *gly* protoplasts pulse-labeled in the presence or absence of monensin (data not shown).

To establish the organelle association of *wt* or *gly* proproteins within the cells, protoplasts were pulse-labeled and gently lysed and separated into soluble (cytosol + vacuolar contents) and organelle (enriched ER/Golgi) fractions. The

molecular forms of radiolabeled barley lectin affinity-purified from soluble (S) or organelle (O) fractions isolated from *wt* or *gly* protoplasts are presented in Figure 8. Both proproteins and mature barley lectins were present in the soluble fraction of *wt* or *gly* protoplasts (Lanes 5 and 7 respectively, Figure 8). However, only the proproteins were readily discernible in organelle fractions isolated from *wt* or *gly* protoplasts (Lanes 6 and 8 respectively, Figure 8). These results demonstrated that *wt* or *gly* proproteins were associated with ER/Golgi compartments. The lower levels of *gly* proprotein evident in soluble and particularly organelle fractions was congruent with a shorter half-life for *gly* proproteins (Figure 7).

DISCUSSION

Barley lectin is a member of a class of vacuolar proteins which are initially synthesized as glycosylated precursors and subsequently processed to mature nonglycosylated proteins by the post-translational cleavage of a COOH-terminal glycopeptide. This class of vacuolar proteins includes the Gramineae lectins and a plant defense-related β-1,3-glucanase of tobacco (Shinshi, *et al.*, 1988). The transient association of an N-linked oligosaccharide side-chain with the proprotein provides a unique opportunity to investigate the functional significance of the N-linked glycan in the post-translational processing and transport of these vacuolar proteins.

Barley lectin was correctly assembled and targeted to vacuoles in transgenic tobacco

The feasibility of expressing a monocot vacuolar protein in a heterologous dicot system was examined by introducing cDNAs encoding the *wt* barley lectin preproprotein under the transcriptional control of the constitutive CaMV 35S promoter into tobacco by *Agrobacteria*-mediated transformation. Analysis of transgenic plants established that the *wt* barley lectin was synthesized as the appropriate 23 kd proprotein in tobacco. The 23 kd *wt* proprotein was correctly modified by the covalent attachment of a 2 kd high mannose oligosaccharide side-chain, post-translationally processed to the mature 18 kd subunit and transported to vacuoles in tobacco analogous to barley embryos (Lerner and Raikhel, 1989). Synthesis of the correct barley lectin proprotein in transgenic tobacco plants was indicative that the signal sequence of this monocot protein was recognized and cleaved by an ER signal peptidase in dicots. Correct utilization of NH_2-terminal signal sequences in heterologous systems is documented for the vacuolar protein PHA (Sturm, *et al.*, 1988) and a chimeric construct employing the signal sequence of the vacuolar storage protein patatin (Iturriaga, *et al.*, 1989). Predicated on the ability to isolate mature barley lectin by affinity chromatography on immobilized GlcNAc, the *wt* proproteins were assembled into the correct dimeric conformation required of an active lectin. In summary, the correct synthesis, assembly, processing and transport of barley lectin to vacuoles in tobacco indicated the existence of a common mechanism for post-translational processing and targeting of proteins to vacuoles in monocots and dicots. A number of storage proteins

and lectins are correctly expressed in seeds of heterologous systems (Beachy, *et al.*, 1985; Sengupta-Gopalan, *et al.*, 1985; Okamuro, *et al.*, 1986; Hoffman, *et al.*, 1987; Sturm, *et al.*, 1988). However, only patatin was shown to be correctly processed in vegetative tissues of tobacco (Sonnewald, *et al.*, 1989). The present study is the first report to demonstrate the correct processing and stable accumulation of a embryo-specific monocot vacuolar protein in tobacco leaves and roots.

Propeptide glycan was not required for correct assembly and transport of barley lectin in transgenic tobacco

The myriad of functions associated with the N-linked oligosaccharides of many mammalian glycoproteins (Olden et al., 1985) indicate that there is no universal role for N-linked glycans. The influence of the barley lectin proprotein glycan on assembly, processing and transport of this protein was investigated by examining the expression of a mutant *gly* barley lectin in transgenic tobacco. The 21 kd nonglycosylated proprotein was correctly synthesized, assembled as an active lectin, transported to vacuoles, and processed to the mature polypeptide in transgenic tobacco analogous to *wt* barley lectin in barley embryos. Although the absence of the propeptide glycan in tobacco plants expressing the *gly* proprotein of barley lectin apparently did not impede the formation of active lectin dimers, it was unknown whether the presence of the glycan or the glycopeptide may influence the rate of assembly of active lectin dimers. Active dimers can actually be assembled from mature nonglycosylated subunits *in vitro* (Peumans, *et al.*,

1982b).

Localization of mature barley lectin derived from the *gly* proprotein in vacuoles of tobacco also demonstrated that the high mannose glycan covalently attached to the COOH-terminal propeptide was not an absolute requirement for the targeting of barley lectin to vacuoles. Similar results are observed for the glycoprotein PHA (Bollini *et al.*, 1985; Voelker, *et al.*, 1989) even though barley lectin is only glycosylated as a precursor and unlike PHA, it is not a glycoprotein in its mature form. The glycans of the barley lectin proprotein and PHA are not essential for processing and targeting of these proteins to vacuoles. Conversely, the glycan of proConA apparently plays a direct role in processing and transport of ConA to vacuoles (Faye and Chrispeels, 1987).

Propeptide glycan affects rate of post-translational processing and transport of barley lectin in transgenic tobacco

To assess the possibility that the N-linked glycan played an indirect role in intracellular processing and transport of barley lectin, pulse-chase labeling and monensin experiments were performed with tobacco protoplasts expressing the *wt* or *gly* barley lectin proproteins. Pulse-chase experiments demonstrated that the glycosylated and unglycosylated proproteins were differentially processed to the mature protein at different rates. The nonglycosylated (*gly*) 21 kd proprotein was processed to the mature 18 kd protein at a rate at least 2-fold faster than the glycosylated (*wt*) 23 kd proprotein. Monensin effectively inhibited the post-

translational processing of both the *wt* and *gly* barley lectin proproteins to the mature subunit in tobacco protoplasts. Fractionation of subcellular components along with the results of the monensin inhibitor experiments established that the proproteins were associated with the Golgi compartment. Lower steady state levels of *gly* proprotein in the Golgi complex relative to *wt* levels indicated that the *gly* proprotein was transported from the Golgi complex faster than the *wt* proprotein.

The protracted rate of processing and transport of the *wt* proprotein relative to the *gly* proprotein implied that deglycosylation of the propeptide preceded processing and transport and was the rate-limiting step in these series of events. The post-translational removal of an internal glycopeptide from proConA is also believed to commence with a deglycosylation step (Bowles, *et al.*, 1986). In contrast to the present study, monensin purportedly has limited effect on the processing of the rice lectin proprotein to the mature protein in developing embryos (Stinissen, *et al.*, 1985). However, similar inhibitory effects by monensin have been observed on the processing of proConA (Bowles, *et al.*, 1986) and pea vicilin proproteins (Craig and Goodchild, 1984).

A model for the role of the glycan in the post-translational processing of barley lectin

The pulse-chase experiments indicated that the N-linked high mannose glycan of the barley lectin propeptide modulates processing and transport of the barley lectin proprotein from the Golgi complex to the vacuoles. The glycan therefore

presumably plays an indirect or negative role in the regulation of processing and transport of barley lectin to vacuoles. We propose that the molecular mechanism by which the glycan regulates these processes relys upon a sequential two-step processing of the proprotein COOH-terminal glycopeptide (Figure 9). Concomitant with the formation of an active lectin dimer, the proprotein assumes a conformation in which the high mannose glycan sequesters the propeptide from the aqueous environment, thereby masking the availability of the propeptide for processing (Figure 9A). This predicted protein configuration is predicated on the conformation of the protein (Wright, 1987), the amphipathic characteristic of the propeptide and the hydrophilic nature of the glycan. In the *trans*-cisternae of the Golgi complex, the glycan is removed post-translationally in a regulated manner from the proprotein. As a consequence, deglycosylation exposes the propeptide to proteases and thereby facilitates further processing and transport of the proprotein (Figure 9B). Therefore, the deglycosylation of the glycopeptide is the rate limiting step in the processing of the proprotein to the mature lectin. However, it can also be postulated that the proprotein COOH-terminal glycopeptide may be removed in a single step. The contribution of the glycan in the processing and transport of this plant vacuolar protein is congruous with the involvement of N-linked glycans in the proteolytic processing and stabilization of many mammalian glycoproteins (Olden, *et al.*, 1985).

Figure 9. Proposed Cascade of Events Involved in the Posttranslational Processing of Barley Lectin.

The processing model schematically depicts one subunit of a barley lectin dimer adapted from the structure of WGA (Wright, 1987). Each of the highly homologous domains of barley lectin is represented by a circle. A high-mannose glycan tree is attached to the sole B-linked glycosylation site (Asn-Ser-Thr) residing within the C-terminal propeptide of barley lectin. Structure of high-mannose type glycan was adapted from Montreuil (1984).

MATERIALS AND METHODS

Modification of Barley Lectin cDNA Flanking Regions

The EcoRI sites flanking the 972 bp cDNA clone (pBLc3) encoding barley lectin (Lerner and Raikhel, 1989) were blunt-ended using DNA Polymerase I Klenow fragment as described in Maniatis, *et al.*, 1982. Following the addition of XbaI phosphorylated linkers (Maniatis, *et al.*, 1982), the cDNA was purified from low-melting point agarose and subcloned (Struhl, 1985) into pUC118 (Vieira and Messing, 1987).

Site-directed Mutagenesis

The N-linked glycosylation site at Asn_{180}-Ser-Thr_{182} in the COOH-terminal propeptide of the barley lectin proprotein (Lerner and Raikhel, 1989) was altered by the converting Asn_{180} (AAC) to a Gly_{180} (GGC) residue by the site-directed mutagenesis method of Kunkel, *et al.* (1987) (see Figure 1A). Site-directed mutagenesis of the barley lectin propeptide was performed using Bio-Rad's Muta-Gene phagemid *in vitro* mutagenesis kit with a mutagenic 16-base synthetic oligonucleotide spanning amino acids Ala_{178} to Thr_{182} (Lerner and Raikhel, 1989) and uracil-containing single-strand DNA prepared in the *dut ung E. coli* strain CJ236. Mutants encoding the altered tripeptide Gly_{180}-Ser-Thr_{182} were identified and selected by ^{35}S-dideoxy sequencing (Sanger *et al.*, 1977) of single-strand DNA prepared from phagemids in the *dut*$^+$ *ung*$^+$ *E. coli* strain MV1193.

Plant Transformation

Both mutated (*gly*) and wild-type (*wt*) barley lectin cDNAs were excised from pUC118 with XbaI and subcloned (Struhl, 1985) into the binary plant expression vector pGA643 (An, *et al.*, 1988). These binary vector constructs were mobilized from the *E. coli* strain DH5α into *Agrobacterium tumefaciens* LBA4404 by triparental mating (Hooykaas, 1988) using the *E. coli* strain HB101 harboring the wide-host range mobilizing plasmid pRK2013. Transconjugates were selected on minimal nutrient plates (An, *et al.*, 1988) containing streptomycin (200 ug/ml), kanamycin (25 ug/ml) and tetracycline (5 ug/ml).

Agrobacteria cells containing the *wt* and *gly* barley lectin cDNAs were introduced into tobacco plants (*Nicotiana tabacum* cv. Wisconsin 38) by the leaf disc transformation method of Horsch, *et al.* (1988). The leaf discs were cocultivated with the *Agrobacteria* for 48 hrs on MS104 plates prior to transfer to MS selection media (Horsch, *et al.*, 1988). After several weeks, shoots were transferred to MS rooting media (Horsch, *et al.*, 1988). At least three independent transformants, maintained as axenic cultures, were subsequently analyzed for each construct.

Nucleic Acid Analysis

Total DNA was isolated from leaf tissue of untransformed and transgenic tobacco plants according to Shure, *et al.* (1983). DNA (12 ug) was restricted with HindIII and fractionated on 1.0% agarose gels prior to transfer to nitrocellulose (Maniatis, *et al.*, 1982). Nitrocellulose filters were hybridized with ^{32}P random-primer-labeled

(Feinberg and Vogelstein, 1983) BLc3 barley lectin cDNA (Lerner and Raikhel, 1989) as described previously (Raikhel, *et al.*, 1988). For gene reconstruction experiments, tobacco genomic DNA was restricted with EcoRI and BLc3 titered at 0.5-, 1.0-, 3.0-, and 5.0-copy equivalents per tobacco (*N. tabacum*) genome (4.8 X 10^9 bp per haploid genome; Zimmerman and Goldberg, 1977). Gene reconstruction blots were hybridized with a radiolabeled Blc3 insert by the random-primer method (Feinberg and Vogelstein, 1983). Filters were exposed to Kodak X-OMAT AR film at -70°C with intensifying screens.

Total RNA was isolated from leaves of untransformed and transgenic tobacco plants as described previously (Wilkins and Raikhel, 1989). Total RNA (25 ug) from each construct was resolved in a 2% agarose/6% formaldehyde gel, transferred to nitrocellulose, and hybridized with the BLc3 cDNA labeled with ^{32}P as described above.

Protein Extraction, Affinity Chromatography, Immunoblots, and ELISA

Barley lectin was purified from acid soluble protein extracts by affinity chromatography on immobilized N-acetylglucosamine columns from transgenic tobacco leaves (500 mg) essentially as described in Mansfield, *et al.* (1988) with the exception that the homogenization buffer consisted of 50 mM HCl containing 1 mM phenylmethylsulfonyl fluoride (PMSF). The affinity-purified lectin was carboxyamidated (Raikhel, *et al.*, 1984), fractionated by SDS-PAGE (Mansfield, *et al.*, 1988), and electroblotted onto nitrocellulose (Towbin, *et al.*, 1979). Barley lectin was detected using anti-WGA polyclonal antiserum (Mansfield, *et al.*, 1988) and

protein A-alkaline phosphatase as described in Blake, *et al.* (1984) using nitroblue tetrazolium as the substrate.

Extracts of acid soluble proteins were assayed using double-bind ELISA (Raikhel, *et al.*, 1984) to quantitate the amount of barley lectin in transgenic tobacco leaves. Crude extracts were prepared by homogenization of tobacco leaves (1.0 g) in 2 ml 50 mM Tris-acetate, pH 5.0, 100 mM NaCl, 1 mM PMSF. The extracts were clarified by centrifugation at 10 krpm for 10 min to remove cellular debris and insoluble material. Barley lectin was detected in crude extracts using guinea pig anti-WGA antiserum and rabbit anti-WGA IgGs conjugated to alkaline phosphatase (Raikhel, *et al.*, 1984). A standard curve, constructed from affinity-purified WGA (E-Y Labs), was used to estimate the level of barley lectin in tobacco leaves. Total protein in the crude extracts was determined by the method of Bradford (1976).

Vacuole Isolation and Enzyme Assays

Protoplasts for vacuole isolation were prepared from leaves of axenic cultured plants. Leaves were digested overnight in an enzyme medium composed of 0.5 M mannitol and 3 mM MES, pH 5.7 containing the same enzymes as described below. Vacuoles were isolated from tobacco protoplasts by ultracentrifugation as described in Guy, *et al.* (1979) with the exception that the isolation buffer was 0.5 M sorbitol and 10 mM HEPES, pH 7.2 and the Ficoll step gradient consisted of 10% and 5% Ficoll. Vacuoles stained with neutral red were collected from the 0%/5% interface and adjusted to 10% Ficoll and subjected to further purification

on a second Ficoll gradient. Vacuoles were collected from the 0%/5% Ficoll interface on a second gradient by flotation of the vacuoles during centrifugation. The vacuoles recovered were counted in a hemocytometer, frozen in liquid nitrogen, and stored at -80°C for biochemical analysis.

Vacuolar-specific enzyme activities of α-mannosidase (Boller and Kende, 1979) and acid phosphatase (Shimomura, *et al.*, 1988) were assayed in protoplast and vacuole fractions by monitoring the release of p-nitrophenol spectrophotometrically from the appropriate substrates. Catalase activity (Aebi, 1974) was measured in protoplast and vacuole fractions as an extravacuolar enzyme marker.

Immunocytochemistry

Leaf tissue from axenic tobacco plants was excised and trimmed into 2 mm^2 pieces. Fixation and immunocytochemistry was performed essentially as described in Mansfield, *et al.* (1988).

Radiolabeling of Tobacco Protoplasts, Endo H Digestion, and Monensin

Protoplasts for labeling were prepared from fully expanded leaves of axenically cultured tobacco plants. Leaves were digested overnight in an enzyme mixture comprised of 0.5% cellulase (Onozuka R10), 0.25% macerozyme R10, and 0.1% BSA in Murashige and Skoog media (Murashige and Skoog,1962) supplemented with 1.0 ug/ml benzyladenine, 0.1 ug/ml napthaleneacidic acid and 0.5 M mannitol (MSA). The yield of protoplasts was quantitated using a hemocytometer counting

chamber.

For pulse-labeling experiments, 1 X 10^5 leaf protoplasts (per well) were incubated in a 24-well Falcon tissue culture plate in 500 ul MSA media supplemented with 48 uCi of ^{35}S-Trans label (ICN ^{35}S *E. coli* hydrolysate labeling reagent containing $\geq$ 70% L-methionine and $\leq$ 15% L-cysteine; 1000-1200 Ci/mmole). The culture plates were incubated in the dark at room temperature with gentle shaking. Two wells or a total of 200,000 protoplasts were labeled for each experiment. Pulse-chase experiments were performed by supplementing the media with 1 mM L-methionine and 0.5 mM L-cysteine 8 to 10 hr after pulse-labeling protoplasts as described above. Following labeling, protoplasts were pooled and collected by centrifugation at 2 krpm for 15 sec at 4°C. The resulting protoplast pellet was suspended in 100 ul of 50 mM Tris-acetate, pH 5.0, 100 mM NaCl and lysed at room temperature for 10 min with gentle agitation following the addition of 100 ul of 1.2 mM dithiothreitol and 1.2 % (v/v) Triton X-100 in Tris-acetate/NaCl. Samples were frozen in liquid N_2 and stored at -70°C.

Endo-β-N-acetylglucosaminidase H (Endo H) digestion of radiolabeled barley lectin was performed at 37°C for 23 hrs in 50 mM Tris-acetate, pH 5.5, 100 mM NaCl, 1 mM PMSF with 4 mU Endo H immediately following affinity-purification of barley lectin from protoplasts pulse-labeled for 12 hr as described above.

For monensin experiments, 500 mM monensin in absolute ethanol was added directly to each well containing tobacco protoplasts to a final concentration of 50 uM monensin, 0.1% ethanol. Absolute ethanol was added to a final concentration of 0.1% in controls. *Wt* or *gly* protoplasts were pretreated in the

presence of ethanol or monensin for 1 hr prior to the addition of ^{35}S-trans label and pulse-labeled for 12 hr. To determine the organelle association of *wt* or *gly* proproteins, organelles were seperated from soluble proteins. A total of 600,000 *wt* or *gly* protoplasts were pooled and gently homogenized in 200 ul of 100 mM Tris pH 7.8, 1 mM EDTA, 12% sucrose (w/w) and separated into soluble and organelle fractions on Sepharose 4B columns (8.0 cm X 1.0 cm) according to Stinissen *et al.* (1985). The Sepharose 4B elution profile of total radioactivity associated with organelles and soluble proteins concurred with previous studies (Stinissen, *et al.*, 1984; Stinissen, *et al.*, 1985; Mansfield, *et al.*, 1988). In addition, NADH cytochrome C reductase activity (Lord, 1983) was primarily associated with the organelle fractions. The samples were adjusted to 0.5% Triton X-100 and stored at -70°C. Following collection of protoplasts by centrifugation (see above), the culture media was recovered from a total of 800,000 protoplasts and contaminating intact protoplasts removed by gravity filtration through a Isolab quick-sep column fitted with a paper filter and a Whatman GF/C glass fiber filter (1.2 um exclusion). Proteins contained in the culture media were precipitated with ammonium sulfate at 60% saturation at 4°C for at least 2 hrs. Precipitated proteins were collected by centrifugation for 10 min at 15 krpm. The protein pellet was resuspended in 200 ul of 50 mM Tris-acetate, pH 5.0, 100 mM NaCl and stored at -70°C. ^{35}S-labeled barley lectin was purified by affinity chromatography, carboxyamidated and analyzed by SDS-PAGE as described above. The SDS-PAGE gels were treated for fluorography as detailed in Mansfield, *et al.* (1988).

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Chapter 3*

A carboxyl-terminal propeptide is necessary for proper sorting of barley lectin to vacuoles of tobacco

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ABSTRACT

Barley lectin is synthesized as a preproprotein with a glycosylated carboxyl-terminal propeptide (CTPP) which is removed prior to or concomitant with deposition of the mature protein in vacuoles. Expression of a cDNA clone encoding barley lectin in transformed tobacco plants results in the correct processing, maturation and accumulation of active barley lectin in vacuoles [Wilkins, Bednarek and Raikhel (1990) The Plant Cell, 2, 301-313]. The glycan of the propeptide is not essential for vacuolar sorting, but may influence the rate of post-translational processing (Wilkins et al., 1990). To investigate the functional role of the CTPP in processing, assembly and sorting of barley lectin to vacuoles, a mutant barley lectin cDNA clone lacking the 15 amino acid CTPP was prepared. The CTPP deletion mutant of barley lectin was expressed in tobacco protoplasts, suspension-cultured cells and transgenic plants. In all three systems the wild-type barley lectin was sorted to vacuoles, whereas mutant barley lectin secreted to the incubation media. Therefore, we conclude that the carboxyl-terminal domain of the barley lectin proprotein is necessary for the efficient sorting of this protein to plant cell vacuoles.

INTRODUCTION

In eukaryotes, proteins of the endoplasmic reticulum (ER), Golgi, lysosomes, vacuoles, plasma membrane, and cell wall are derived from a subset of proteins that enter the secretory pathway. Proteins are targeted to the secretory pathway by a N-terminal hydrophobic signal sequence which mediates a transmembrane translocation from the cytosol to the lumen of the endoplasmic reticulum (ER). Following proteolytic cleavage of the signal sequence, some secretory proteins undergo further post-translational processing in the ER and Golgi network (Blobel and Dobberstein, 1975). Proteins traversing the secretory pathway are believed to be sorted to their respective compartments by selective retention or targeting information contained in their molecular structures (Rothman, 1987). Proteins lacking specific sorting determinants follow a default pathway and are consequently secreted toward the cell surface (Rothman, 1987; Wieland et al., 1987; Dorel et al., 1989; Denecke et al., 1990).

A secondary sorting signal that mediates a targeting process involves either a post-translational modification of the protein or depends upon primary, secondary, or tertiary structural elements within the polypeptide (Verner and Schatz, 1988). The most well characterized sorting process is the mannose-6-phosphate dependent sorting of mammalian lysosomal enzymes (Kornfeld, 1987). The active sorting of these enzymes to the lysosome is dependent on the modification of specific glycans with mannose-6-phosphate and the binding of the modified glycan to mannose-6-phosphate receptors in the trans-Golgi network

(reviewed in Kornfeld and Mellman, 1989). However, there is evidence for the existence of a mannose-6-phosphate independent system for the sorting of mammalian lysosomal enzymes (Gabel et al., 1983).

In yeast and plants, N-linked glycans are not necessary for the correct transport and sorting of secretory proteins to vacuoles (Stevens et al., 1982; Voelker et al., 1989; Wilkins et al., 1990; Sonnewald et al., 1990). Therefore, it appears that targeting of proteins to vacuoles in yeast and plants is independent of post-translational modifications to oligosaccharide side chains and may be dependent upon elements within the polypeptide. Through deletion analysis and the study of hybrid proteins consisting of amino-terminal segments of the yeast vacuolar carboxypeptidase Y (CPY) fused with the secreted enzyme invertase, it was demostrated that the sorting signal of CPY is contained within the amino-terminal propeptide of CPY (Johnson et al., 1987; Valls et al., 1987). Further mutational analysis of an amino-terminal segment of this propeptide determined that the tetrapeptide QRPL functions as a vacuolar sorting signal. Interestingly, the context in which the QRPL sequence is presented affects the efficiency of targeting, inferring the involvement of secondary structural elements in the sorting mechanism of CPY (Valls et al., 1990). A sorting determinant was identified in the amino-terminal propeptide of another yeast vacuolar enzyme, proteinase A (Klionski et al., 1988), which is sufficient to redirect the normally secreted enzyme invertase to the yeast vacuole. However, currently no concensus sequence or common structural determinant has been demonstrated for targeting of yeast vacuolar proteins, suggesting that a diverse array of factors are involved in the

sorting process.

It has been also shown that the plant vacuolar protein phytohemagglutinin-L (PHA), a lectin of *Phaseolus vulgaris*, is correctly processed and sorted to the yeast vacuole (Tague and Chrispeels, 1987), and will redirect a fusion protein containing the secreted form of yeast invertase to the yeast vacuolar compartment (Tague and Chrispeels, 1989). Deletion analysis localized the vacuolar sorting domain within the amino-terminal portion of mature PHA (Tague et al., 1990). This domain contains a yeast-like targeting tetrapeptide sequence LQRD, and is sufficient to target PHA-invertase hybrid proteins to the yeast vacuole (Tague et al., 1990). It should be noted, however, that the same PHA-invertase fusion proteins were not succesfully targeted to vacuoles in *Arabidopsis thaliana* protoplasts (Chrispeels, 1991). Therefore this sorting determinant contains enough information for vacuolar sorting in yeast, but appears to lack the necessary information for efficient targeting in plants, suggesting that vacuolar sorting signals in yeast and plants are dissimilar.

We are interested in the molecular mechanisms regulating vacuolar sorting of Gramineae lectins. Gramineae lectins are vacuolar proteins which are initially synthesized as glycosylated 23 kD polypeptides which dimerize within the lumen of the ER to form an active GLcNAc-binding proprotein (Mansfield et al., 1988). During transport or after arrival in the vacuoles, the glycosylated carboxyl-terminal propeptide (CTPP) is removed from the proprotein to yield the mature lectin. We are using a transgenic system to define and characterize the molecular mechanisms that mediate the processing and vacuolar sorting of barley lectin. As

a first step, we have demonstrated that barley lectin is correctly assembled, processed and targeted to vacuoles in transgenic tobacco (Wilkins et al., 1990). A functional analysis of the carboxyl-terminal propeptide glycan revealed that although the glycan is not essential for processing and vacuolar sorting of probarley lectin in tobacco, it's presence does modulate the rate of processing of the propeptide (Wilkins et al., 1990).

Although the primary sequences of the carboxyl-terminal propeptides of wheat germ agglutinin (WGA), rice lectin and barley lectin are not conserved, these CTPPs share the potential to form amphipathic α-helices (Wilkins and Raikhel, 1989). Amphipathic α-helices are believed to function as targeting signals in mitochondrial protein import as well as mediating other protein-protein interactions (Verner and Schatz, 1988). In this paper we extended the analysis of the functional role of the carboxyl-terminal propeptide by examining the assembly and sorting of a barley lectin mutant lacking the carboxyl-terminal propeptide. We have established transient expression and stably transformed suspension-cultured cells systems in addition to using transgenic plants to facilitate the analysis of the vacuolar sorting of barley lectin. Using the three systems we have determined that the 15 amino acid carboxyl-terminal propeptide domain is necessary for correct sorting of barley lectin to the vacuole.

RESULTS

Deletion of the Carboxyl Terminal Propeptide of proBarley Lectin

The barley lectin cDNA clone (pBlc3) (Lerner and Raikhel, 1989) encodes a polypeptide containing a 26 amino acid signal sequence and a 186 amino acid proprotein. In the lumen of the ER the signal sequence is cleaved and the polypeptide is co-translationally is glycosylated. The proprotein consists of four highly homologous domains of 43 amino acids each and a 15 amino acid carboxyl-terminal propeptide (CTPP) which contains a N-linked high mannose glycan. Prior to or concomitant with deposition of mature barley lectin in the vacuole, the glycosylated 15 amino acid CTPP is cleaved to yield the dimer consisting of two identical 18 kD subunits. To investigate the role of the CTPP in the assembly and sorting of barley lectin to vacuoles, a mutant barley lectin cDNA clone lacking the 15 amino acid CTPP was prepared. The CTPP coding region [nucleotide (nt) 607 to 651 of the cDNA clone pBlc3 (Lerner and Raikhel, 1989)] was deleted by site-directed mutagenesis (Kunkel et al. 1987). A synthetic oligonucleotide (see Methods) complementary to regions flanking the CTPP coding sequence was utilized as a primer to initiate second-strand synthesis of a mutant barley lectin clone lacking nt # 607 to 651. The CTPP barley lectin deletion mutant cDNA was subcloned into the binary plant expression vector pGA643 under transcriptional control of the 35S cauliflower mosaic virus promoter (An et al. 1988). Constructs containing the CTPP deletion mutant of barley lectin are designated by the code *ctpp* (Figure 1). We have previously designated pGA643 constructs

containing the barley lectin cDNA by the code *wt* (Figure 1) (Wilkins et al., 1990).

Transient Protein Synthesis of Active *wt* and *ctpp* Barley Lectin in Tobacco Suspension-Cultured Cell Protoplasts

Barley lectin is localized in vacuoles/protein bodies of embryonic and adult root cap cells of barley (Mishkind et al., 1983; Lerner and Raikhel, 1989). We have previously demonstrated that barley lectin is also correctly processed and targeted to vacuoles in transgenic tobacco cells (Wilkins et al., 1990). To determine whether the *ctpp* mutant of barley lectin was synthesized and assembled into an active lectin in tobacco, *ctpp* constructs were transiently expressed in tobacco suspension-cultured cell (NT) protoplasts. *Wt* and *ctpp* pGA643 constructs were introduced into NT protoplasts via polyethylene glycol treatment (Negrutiu et al., 1987) and the protoplasts were pulse-labeled for 12 hours in the presence of a mixture of ^{35}S-labeled methionine and cysteine (^{35}S-met/cys) (see Methods). Protein extracts prepared from the labeled protoplasts and incubation media were fractionated on immobilized N-acetylglucosamine (GlcNAc). The affinity purified fractions were analyzed under denaturing conditions by SDS-PAGE and fluorography as shown in Figure 2. Two polypeptides corresponding to the 23 kD proprotein and 18 kD mature subunit of barley lectin were present in the pulse-labeled NT protoplast expressing the *wt* construct (lane 2, Figure 2). Radiolabeled barley lectin was not detected in the incubation media of NT protoplasts expressing *wt* barley lectin (lane 5, Figure 2). Similarly, barley lectin is not detected in the incubation media of ^{35}S-met/cys labeled leaf protoplasts from *wt*

Figure 1. Organization of the Wild-Type (*wt*) and Carboxyl-Terminal Mutant (*ctpp*) Barley Lectin cDNAs.

Site-directed mutagenesis was used to prepare a *ctpp*- barley lectin construct. The *wt* and *ctpp* barley lectin cDNA clones were subcloned into the plant expression vector pGA643 (An et al., 1988). *Wt* barley lectin encodes a polypeptide containing a 26 amino acid signal sequence, followed by a 186 amino acid proprotein (amino acids +1-186). The 15 amino acid CTPP of probarley lectin (amino acids 172-186) contains an N-linked high mannose type glycan as depicted by the branched structure.

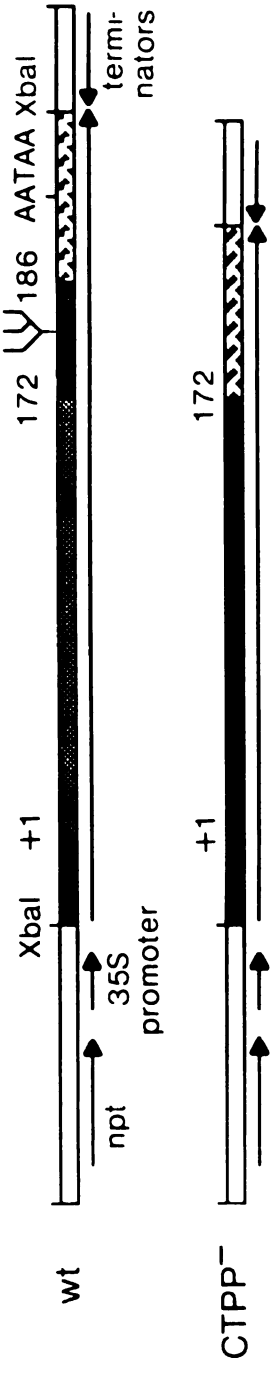
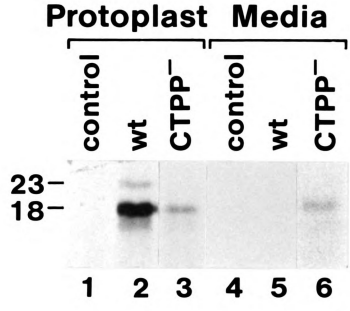

wt
CTPP⁻
XbaI
+1
35S
promoter
npt
172
186
AATAA
XbaI
termi-
nators
172
+1

Figure 2. Protein Gel Blot Analysis of Transiently Expressed *wt* and *ctpp*⁻ Barley Lectin cDNA Constructs.

Protoplasts from tobacco suspension cultured cells were transiently transformed with *wt* and *ctpp*⁻ pGA643 constructs via direct gene transfer using polyethylene glycol (Negrutiu et al., 1987) and were pulse-labeled for 12 hr. Radiolabeled barley lectin was affinity purified from both protoplasts and incubation media and seperated by SDS-PAGE as described in Methods. Lanes 2 and 5 represent radiolabeled barley lectin extracted from *wt* protoplasts and corresponding incubation media, respectively. Lanes 3 and 6 are radiolabeled barley lectin from *ctpp*⁻ protoplasts and incubation media, respectively. Lanes 1 and 4 refer to extracts prepared from protoplasts treated in the absence of plasmid DNA, and are the negative controls. The sizes of the barley lectin precursor (23 kD) and mature barley lectin (18 kD) are shown on the left.


Protoplast
Media
control
wt
CTPP⁻
control
wt
CTPP⁻
23–
18–
1
2
3
4
5
6

transgenic tobacco (Wilkins et al., 1990). Only the mature 18 kD subunit of barley lectin was present in the affinity purified extracts from pulse-labeled *ctpp*⁻ NT protoplasts (lane 3, Figure 2). Deletion of the carboxyl-terminal propeptide resulted in the appearance of radiolabeled barley lectin in the incubation media of the protoplasts transiently expressing the *ctpp*⁻ construct (lane 6, Figure 2). These results support the observation (Peumans et al., 1982) that the subunits of barley lectin do not require the CTPP to correctly dimerize *in vitro*. However, the CTPP does appear to be necessary for proper sorting of active barley lectin to the vacuole.

Transformation of *wt* and *ctpp*⁻ Constructs into Tobacco Suspension-Cultured Cells and Plants

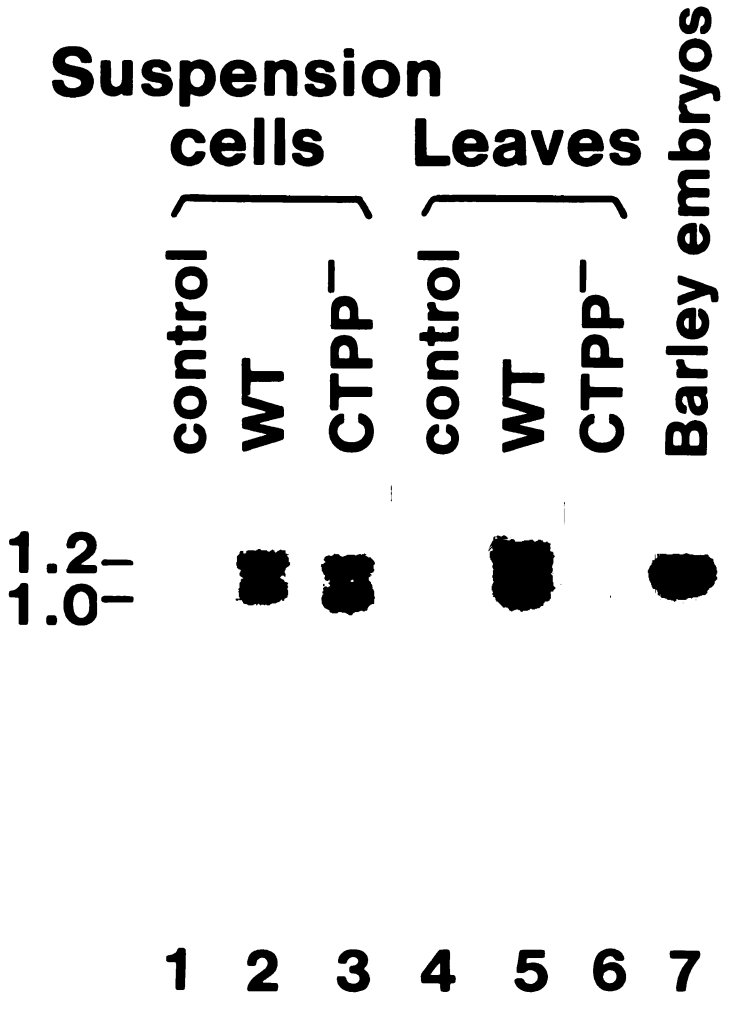
To further investigate the role of the carboxyl-terminal propeptide in the sorting of barley lectin to vacuoles, tobacco plants and suspension-cultured cells were stably transformed with *wt* and *ctpp*⁻ constructs. In our previous experiments, we have analyzed the expression and intracellular localization of barley lectin in transgenic tobacco plants. However, suspension-cultured cells offer many advantages for the analysis of protein sorting. NT cells can be readily transformed a high frequency by co-cultivation with *A. tumefaciens* and kanamycin-resistant transformants can be analyzed within 7-8 weeks after selection. The individual nature of suspension-cultured cells and their immediate contact with the surrounding media allows for the direct analysis of protein secretion from transformed cells.

NT cells were transformed by cocultivation with *Agrobacterium tumefaciens*

containing *wt* or *ctpp*⁻ pGA643 constructs according to the method of An (1985). Kanamycin-resistant calli expressing barley lectin were designated NT-*wt* and NT-*ctpp*⁻, respectively. Kanamycin resistant tobacco plant transformants containing the CTPP deletion mutant of barley lectin were generated as described in Wilkins et al. (1990). Transgenic plants expressing the mutant barley lectin were designated by the code W38-*ctpp*⁻. Tobacco transformants expressing *wt* barley lectin described in Wilkins et al. (1990) were designated by the code W38-*wt*.

The steady state levels of *wt* and *ctpp*⁻ barley lectin mRNA in transgenic NT cells and tobacco plants were compared by RNA gel blot analysis. Barley lectin mRNA was detected by hybridization with ^{32}P-labeled Blc3 insert (Lerner and Raikhel, 1989). Figure 3 depicts the relative levels of barley lectin mRNA in total RNA isolated from *wt* and *ctpp*⁻ transformants examined in this paper. Two mRNA species of 1.2kb and 1.0 kb were observed in total RNA from *wt* transformants (lanes 2 and 5, Figure 3). Two slightly smaller mRNA species of 1.15 kb and 1.05 kb were detected in total RNA from *ctpp*⁻ transformants (lanes 3 and 6, Figure 3). No hybridization of ^{32}P-labeled barley lectin cDNA to total RNA from untransformed NT cells and tobacco leaves was detected at high stringency hybridization conditions (lanes 1 and 4, Figure 3). The relative levels of *ctpp*⁻ barley lectin mRNA were three to four fold lower than corresponding *wt* mRNA in transformants as determined by scanning densitometry (lanes 5 and 6, Figure 3). This disparity in the relative mRNA levels was also manifested in the steady state accumulation of barley lectin protein in *wt* and *ctpp*⁻ transgenic plants (see below).

Figure 3. Accumulation of Steady-State mRNA Levels of Barley Lectin in Tobacco Suspension-cultured Cells (NT) and in Transgenic Tobacco. RNA gel blot analysis of total RNA from the tobacco suspension-cultured cells (NT) or tobacco leaves. Total RNA (25 μg) from untransformed NT cells (lane 1), transformed NT cells containing *wt* (lane 2) or *ctpp*⁻ (lane 3) barley lectin cDNA constructs, untransformed tobacco (W38) (lane 4), transgenic tobacco plants containing *wt* (lane 5) or *ctpp*⁻ (lane 6) barley lectin cDNA constructs, were separated by electorphoresis on a 2% agarose/6% formaldehyde gel and analyzed as described in Methods. Total RNA (10μg) isolated from developing barley embryos (lane 7) serves as positive control. The sizes of the *wt* barley lectin mRNA species(in kilobases) are shown on the left.


Suspension cells
Leaves
control
WT
CTPP⁻
control
WT
CTPP⁻
Barley embryos
1.2
1.0
1 2 3 4 5 6 7

The CTPP Mutant of the Vacuolar Barley Lectin is Secreted in Transgenic Tobacco Suspension-Cultured Cells and Plants

Evidence to date suggests that secretory proteins destined for the vacuoles/lysosomes of plant, mammalian and yeast cells require specific targeting signal(s) for proper sorting (reviewed in Chrispeels, 1991). Secretory proteins which lack or have altered targeting signals cannot be recognized by the vacuolar protein sorting machinery, will be secreted via the default pathway as a consequence (Rothman, 1987; Wieland et al., 1987; Dorel et al., 1989; Denecke et al.,1990).

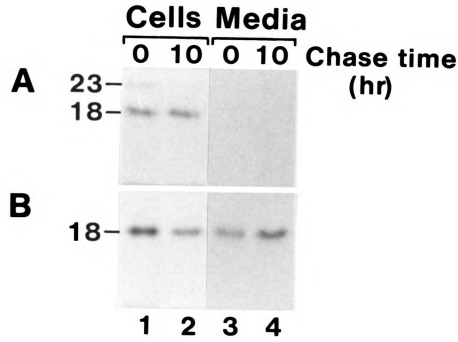
To examine the processing, assembly, and sorting of barley lectin in tobacco suspension-cultured cells, NT-*wt* and NT-*ctpp*⁻ cells were pulse-labeled for 6 hours in the presence of ^{35}S-met/cys and chased for additional 10 hours in the presence of unlabeled methionine and cysteine (met/cys). Crude intracellular and extracellular protein extracts were fractionated on immobilized GlcNAc. As shown in figure 4, radiolabeled barley lectin was analyzed by SDS-PAGE and fluorography. The 23 kD polypeptide and mature 18 kD subunits of barley lectin were readily discernible in NT-*wt* cells (lane 1, Figure 4A). During the 10 hour chase period, the 23 kD polypeptide became almost undetectable. The disappearance of the precursor was accompanied by a corresponding increase in the level of the intracellular 18 kD mature subunit (lane 2, Figure 4A). Neither the labeled precursor nor the mature polypeptide of barley lectin were present in the NT-*wt* incubation media during the 10 hour chase period (lanes 3 and 4, Figure 4A). However, the 18 kD mature polypeptide of barley lectin was detected

Figure 4. Secretion of Barley Lectin from Tobacco Suspension Cultured Cells Transformed with *wt* and *ctpp* Constructs.

(A.) NT cells expressing *wt* barley lectin and incubation media.

(B.) NT cells expressing *ctpp* barley lectin and incubation media.

Tobacco cells were pulse-labeled for 6 hr and chased for 10 hr. Radiolabeled barley lectin was affinity purified on immobilzed GlcNAc from protein extracts of the labeled cells and incubation media. Affinity purified proteins were analyzed by SDS-PAGE on 12.5% polyacrylamide gels and fluorography. The molecular mass (in kilodaltons) of the *wt* precursor (23 kD) and mature subunit (18 kD) are shown on the left.


Cells
Media
0 10 0 10
Chase time (hr)
A
23–
18–
B
18–
1 2 3 4

in both the NT-*ctpp*⁻ cells (lane 1, Figure 4B) and incubation media (lane 3, Figure 4B). During the 10 hour chase period, there was a decrease in the level of intracellular 18 kD polypeptide (lane 2, Figure 4B) and a corresponding increase in the amount of 18 kD barley lectin subunit in the media (lane 4, Figure 4B). Radiolabeled 18 kD subunit was still present in the NT-*ctpp*⁻ cells after a 10 hour chase.

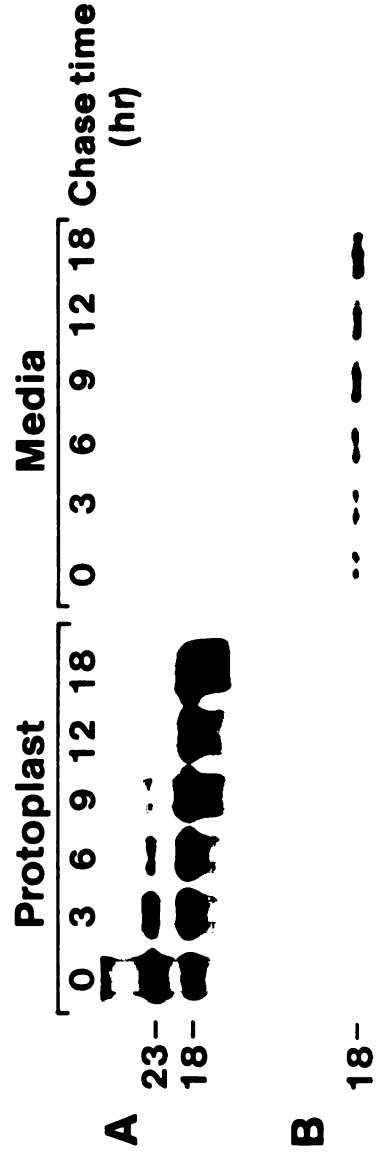
To extend the analysis of *ctpp*⁻ barley lectin secretion, W38-*wt* and W38-*ctpp*⁻ leaf protoplasts were pulse-labeled for 10 hours in the presence of ^{35}S-met/cys. Labeled proteins were chased with unlabeled met/cys for an additional 18 hours. Radiolabeled barley lectin was affinity purified from crude protein extracts of protoplasts and incubation media at specified intervals during the chase period, as shown in figure 5, and analyzed as described above. At the start of the chase (Ohr, Figure 5A), both the 23 kD proprotein and mature 18 kD subunit were present in *wt* protoplasts. During the course of the chase, the level of the 23 kD precursor gradually decreased whereas the level of 18 kD mature subunit correspondingly increased. However, some 23 kD precursor was still visible after 18 hours of chase in the *wt* protoplasts (18 hr, Figure5A), indicating the continued low level incorporation of labeled amino acids into newly synthesized polypeptides. After 10 hours of pulse-labeling, mature 18 kD polypeptide derived from CTPP barley lectin accumulated to higher levels in the incubation media than intracellularly (Figure 5B). Over the course of the chase the level of the intracellular 18 kD subunit decreased and concomitantly increased in the *ctpp*⁻ protoplast incubation media (Figure 5B). After 18 hours of chase, some 18 kD polypeptides

Figure 5. Pulse-Chase Labeling Experiments of Tobacco Protoplasts Isolated from Transformed Tobacco Plants Expressing *wt* or *ctpp* Barley Lectin..

(A.) Tobacco protoplast expressing *wt* barley lectin and incubation media.

(B.) Tobacco protoplast expressing *ctpp* barley lectin and incubation media.

Protoplasts were pulse labeled for 10 hr and chased for 18 hr. Protien extracts were prepared from the protoplasts and incubation media at specified intervals (hr) as indicated during the chase. Radiolabeled barley lectin was affinity purified and analyzed as in **Figure 4**. The molecular mass (in kilodaltons) of the *wt* barley lectin precursor (23 kD) and mature subunit (18 kD) are displayed on the left.


Protoplast
Media
0 3 6 9 12 18
0 3 6 9 12 18
Chase time (hr)
A
23–
18–
B
18–

were still associated with the protoplast fraction. The subcellular distribution of the residual 18 kD *ctpp*⁻ polypeptide was examined by organelle fractionation as described in Wilkins et al. (1990). Vacuoles were isolated from labeled W38-*wt* and W38-*ctpp*⁻ protoplasts as described in Wilkins et al. (1990). The vacuoles were lysed by osmotic shock and the soluble vacuolar proteins were fractionated on immobilized GlcNAc. Affinity purified radiolabeled barley lectin was visualized by SDS-PAGE and fluorography. The 18 kD barley lectin subunit was only discernible in the vacuole preparation from *wt* protoplasts after 60 hours of exposure and not in vacuoles from *ctpp*⁻ transformants (data not shown). However, after a 14 day exposure of the same gel, a another band corresponding to the 23 kD precursor was visable in the vacuolar fraction of W38-*wt* protoplasts and an 18 kD polypeptide could be seen in vacuoles isolated from W38-*ctpp*⁻ protoplasts (data not shown). The appearance of the 23 kD polypeptide suggests that the *wt* vacuole preparation is contaminated with ER and Golgi organelles. Therefore, it is difficult to assess whether the presence of radiolabeled barley lectin in the *ctpp*⁻ vacuoles is the result of some remaining vacuolar targeting of barley lectin lacking the CTPP or, whether it results from contamination of the vacuole preparation by ER and Golgi compartments. Another possibility is that the chase was incomplete and that some low level incorporation of ^{35}S-met/cys into newly synthesized preprobarley lectin still continued.

In this experiment, a low level of the 23 kD proprotein was discernable in the incubation media of W38-*wt* protoplasts. The absence of any detectable 18 kD subunit in the media indicates that the presence of the glycosylated proprotein

is not due to protoplast breakage.

DISCUSSION

The vacuole is a multifunctional organelle important in the regulation and maintenance of plant cell growth and development. Recently, much research has been directed toward understanding the mechanisms controlling the sorting and delivery of secretory proteins to vacuoles. To understand the mechanisms involved in protein sorting to vacuoles, it is necessary to identify and characterize the sorting signals from various vacuolar proteins with different functional and structural properties. We have established both transgenic and transient gene expression systems to investigate the mechanisms of post-translational processing and sorting of barley lectin to plant cell vacuoles. In transgenic tobacco, barley lectin is correctly synthesized as a glycosylated proprotein and assembled as an active GlcNAc-binding dimer in the ER (Wilkins et al., 1990). The proprotein is transported through the Golgi apparatus and is processed to its mature form by removal of a glycosylated 15 amino acid CTPP before or concomitant with deposition of the mature protein in the vacuoles of tobacco leaves (Wilkins et al., 1990). The rate of processing of the precursor is retarded by the presence of an N-linked high mannose glycan on the CTPP. However, the glycan is not required for vacuolar targeting of barley lectin (Wilkins et al., 1990).

Barley lectin and wheat germ agglutinin (WGA) are presumed to share a conserved molecular structure (Wilkins et al., 1990). Extensive X-ray

crystallographic and sequence analysis of mature WGA has revealed that identical 18 kD subunits are composed of four highly 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 region(s) which extend from the surface of the molecule. The lectins from barley, wheat and rice are all initially synthesized as high molecular weight proproteins with glycosylated CTPPs (Raikhel and Wilkins, 1987; Mansfield et al., 1988; Lerner and Raikhel, 1989; Wilkins and Raikhel, 1989). These CTPPs (Figure 6) are predicted by computer analysis of protein secondary structure to form amphipathic α-helices (Wilkins and Raikhel, 1989). In contrast to tightly folded and compact lectin domains, the CTPP may be more exposed on the surface of the lectin dimer and free to interact with other proteins or protein complexes. Based on examination of the compact WGA crystal structure and predicted conformation of the precursor CTPP, we have hypothesized that the CTPP, may function as a sorting determinant for targeting of barley lectin to the vacuole.

To examine the role of the carboxyl-terminal propeptide in vacuolar sorting of barley lectin, we have expressed a CTPP deletion mutant of barley lectin in tobacco protoplasts, transgenic suspension-cultured cells and transgenic plants. Using these three different systems, deletion of the barley lectin CTPP resulted in the secretion of the mature GlcNAc-binding protein. Low levels of radiolabeled barley lectin were still detected intracellularly after 18 hours of chase with unlabeled met/cys. We have not established whether the remaining intracellular barley lectin was sorted to the vacuole without a CTPP or whether it remains sequestered within

the secretory pathway. It has previously been demostrated that deletion of the propeptide region containing the sorting determinant of the yeast vacuolar proteinase A, still resulted in some small fraction of the protein being transported to the vacuole (Klionsky et al., 1988). It is clear however, that barley lectin is missorted if it is synthesized without the CTPP and the data strongly suggests that the carboxyl-terminal propeptide is necessary for efficient sorting of barley lectin to vacuoles.

Experiments are in progress to determine whether the CTPP is also sufficient to target a nonvacuolar reporter protein to vacuoles. If the CTPP is not sufficient to redirect a fusion protein, other regions within the mature protein may be required in conjunction with the CTPP for proper sorting of barley lectin to vacuoles. The overall tertiary structure of the barley lectin proprotein may also affect the proper presentation and recognition of the CTPP by the vacuolar protein sorting apparatus. In addition, the Gramineae lectin precursors are dimers consisting of two identical subunits, each with its own CTPP. This unique structure raises the question of whether a single CTPP will be sufficient for sorting of these vacuolar proteins.

Many vacuolar proteins are synthesized as larger precursors and are processed to their mature form prior to or upon arrival of the proprotein to vacuoles. Similar to the Gramineae lectins, the vacuolar isoforms of β-1,3-glucanases of *Nicotiana tabacum* and *N. plumbaginifolia* are initially synthesized as glycosylated precursors and processed into their mature forms by the removal of a glycosylated carboxyl-terminal propeptide (Shinshi et al., 1988; Van Den

Bulcke et al., 1989). By analogy to the barley lectin CTPP, the β-1,3-glucanase CTPPs may be necessary for vacuolar sorting. The primary amino acid sequences of the Gramineae lectin and the tobacco β-1,3-glucanase CTPPs are not conserved (Figure 6), however these CTPPs all contain a utilized N-linked glycosylation site and have an overall negative charge due to acidic amino acids. If the CTPP from β-1,3-glucanase is necessary for vacuolar sorting, features such as the acidic nature of the glycopeptide and/or secondary structure may be important in defining the sorting determinant.

Interestingly, in contrast to the Gramineae lectins, distinct extracellular isoforms of the β-1,3-glucanases have been identified in *N. plumbaginifolia* (Van Den Bulcke et al., 1989). It is not known whether the extracellular forms were synthesized similarly to the intracellular forms with or without a CTPP. Recently, another β-1,3-glucanase cDNA from *N. tabacum* (Neale et al., 1990) was isolated. This clone is homologous with the vacuolar β-1,3-glucanase cDNA isolated by Shinshi et al. (1988), however it lacks the region encoding the CTPP (Neale et al., 1990). Localization of the β-1,3-glucanase encoded by this cDNA clone is eagerly awaited.

Mechanisms of Barley Lectin Sorting

In mammalian cells, sorting of lysosomal enzymes tagged by mannose-6-phosphate interact with the mannose-6-phosphate receptor system in the trans-Golgi and are segregated into vesicles destined for the lysosome (Kornfeld and Mellman, 1989). Likewise in yeast, the soluble vacuolar protein CPY is believed to

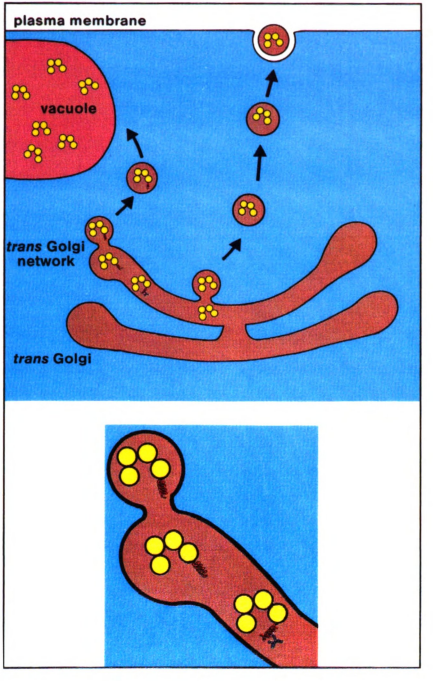
Figure 6. Amino Acid Sequence Comparison of Carboxyl-Terminal Propeptides of Gramineae Lectins and Tobacco β-1,3-glucanases.

The primary amino acid sequences of the carboxyl-terminal propeptides of the Gramineae lectins (add ref) and the intracellular isoforms of *N. tabacum* (Shinshi et al., 1988) and *N. plumbaginifolia* (De Loose et al., 1988) β-1,3-glucanase with the exception of the WGA isolectins and barley lectin are not conserved. Outlined asparagine residues (ℕ) denote utilized N-linked glycosylation sites. Acidic residues (E=glutamic acid, D=Aspartic acid) are highlighted in bold type.

Protein	C-terminal extension
Barley Lectin	VFAEAIAANSTLVAE
WGA-A	VFAEAITANSTLLQE
WGA-B	VFAEAIATNSTLLAE
WGA-D	VFAGAITANSTLLAE
Rice Lectin	DGMAAILANNQSVSFEGIIESVAELV
N. tabacum β-1,3-glucanase	VSGGVWDSSVETNATASLVSEM
N. plumbaginifolia β-1,3-glucanase	FSDRYWDISAENNATAASLISEM

be sorted in a late Golgi compartment (Valls et al., 1987). Studies with the inhibitor monensin on the processing of barley lectin also suggest that sorting of the lectin precursor is a late Golgi event (Wilkins et al., 1990). Monensin primarily disrupts protein transport and sorting in the trans-Golgi (Tartakoff, 1983; Chrispeels, 1983). In the presence of monensin, cleavage of the CTPP and transport of the barley lectin precursor from the Golgi is blocked (Wilkins et al., 1990). The sorting apparatus for the barley lectin precursor is therefore presumably associated with the trans-Golgi compartment. In this paper, we have demonstrated that the CTPP is necessary for sorting of barley lectin proproteins to plant vacuoles. By analogy with the receptor-mediated lysosomal protein sorting system, we propose that the CTPP is recognized by a sorting system and that the proprotein is segregated into vesicles destined for the vacuoles in the trans-Golgi network (Figure 7). The final step in the maturation of barley lectin has not been precisely characterized. It remains unknown whether the carboxyl-terminal propetide is cleaved from the precursor while enroute to or after deposition of the mature lectin in the vacuoles. The N-linked high mannose glycan present on the proprotein CTPP slows the rate of processing of the proprotein, possibly by masking the availability of the CTPP for processing (Wilkins et al., 1990). The glycan is not required for sorting of barley lectin to vacuoles (Wilkins et al. 1990). It has been suggested that the function of some glycans may be to mask "accidental" targeting signals (Tague et al., 1990). Deglycosylation of the carboxyl-terminal glycopeptide may be required for recognition of the CTPP by the sorting machinery and the subsequent processing of the proprotein. This invokes a model whereby the glycosylated

Figure 7. A Model for Barley Lectin Sorting in the trans-Golgi Network. The schematic representation of one subunit of a barley lectin dimer was adapted from crystal structure of WGA (Wright, 1987). Each of the four highly homologous domains of barley lectin is represented by a circle. The glycosylated carboxyl-terminal propeptide is depicted as a spiral to denote the predicted amphiphatic α-helical structure of the peptide and the structure of the N-linked high mannose type glycan was adapted from Montreuil (1984).


plasma membrane
vacuole
trans Golgi network
trans Golgi

proprotein is processed to the mature lectin by a two step procedure (see Wilkins *et al.*, 1990). An intermediate processing form of barley lectin containing a deglycosylated CTPP has not yet been identified, suggesting that the next processing step, the removal of the CTPP, is very fast, or processing of the glycosylated CTPP actually occurs in a single step. The later model suggests that glycosylation of the CTPP does not affect its recognition as a sorting determinant.

Proteins lacking or failing to present an appropriate sorting determinant to the sorting apparatus would be secreted by default from the Golgi via secretory vesicles. Similarly, overproduction of a vacuolar protein may saturate the sorting pathway, therby resulting in the secretion of the protein via the default pathway as has been hypothesized by Stevens et al. (1986). Secretion of barley lectin in W38-*wt* protoplasts to the incubation media presumably resulted from the overproduction of the 23 kD glycosylated proprotein. Overproduction of the proprotein may have saturated either the system which deglycosylates the proprotein or the sorting apparatus which recognizes the CTPP and targets barley lectin to plant vacuoles.

MATERIALS AND METHODS

Preparation of *ctpp* constructs

Nucleotides 607 to 651 of the barley lectin cDNA (Lerner and Raikhel, 1989), encoding the carboxyl-terminal propeptide of the barley lectin proprotein, were deleted by site directed mutagenesis (Kunkel et al., 1987). Uracil-containing single stranded *wt* barley lectin cDNA (Wilkins et al., 1990) was prepared from

bacteriophage M13KO7 grown on the host *dut ung* F^+ *Escherichia coli* strain CJ236 harboring *wt* barley lectin cDNA in pUC118 (Vieira and Messing, 1987). The synthetic mutagenic oligonucleotide, 5'-CGGCGGCTGCGACGGT/TGATGATCTTGCTAATGGCAG-3'(nt 591 to 606/nt 652 to 672), was annealed to the uracil containing single stranded template and used to prime second-strand synthesis by T4 DNA polymerase (New England BioLabs). The mutagenic primer was prepared by the MSU Macro Molecular Facility. Carboxyl-terminal propeptide deletion mutants of barley lectin were identified and selected by ^{35}S-dideoxy sequencing (Sanger et al., 1977) of single stranded DNA prepared from *dut*$^+$ *ung*$^+$ F^+ *E. coli* strain MV1193 transformed with second-strand reaction products. The *ctpp*$^-$ barley lectin cDNA was excised from pUC118 with XbaI (New England Biolabs), subcloned (Struhl, 1985) into the binary plant expression vector pGA643 (An et al., 1988) and mobilized into the *E. coli* strain DH5α. Unless otherwise noted, all standard recombinant DNA techniques used in this paper are as described by Maniatis et al. (1982). All reagents, unless specified were purchased from Sigma.

Suspension-Cell and Shoot Tissue Culture

Nicotiana tabacum suspension cells were maintained in liquid Murashige and Skoog medium (MS) (Murashige and Skoog, 1962) supplemented with 0.2mg/L 2,4-D (MS 0.2mg/L 2,4-D) at 28° C with shaking on a gyratory shaker at 150 rpm. Suspension cells were subcultured weekly with a 5% inoculum of fresh media. Axenic shoot cultures of *Nicotiana tabacum* (cv Wisconsin 38) were maintained

and propagated by node cuttings on solid MS.

Transient Gene Expression System

The NT protoplasts were transformed via the PEG mediated DNA uptake method of Negrutiu et al. (1987). Protoplasts were prepared from 3 day NT suspension cell cultures. NT cells were collected by centrifugation at 50 X g for 5 min at room temperature. The cell pellet was resuspended and digested in MS 0.2mg/L 2,4-D with 1.0% cellulase Onozuka R10, 0.5% macerozyme R10 (Yakult Honsha Co., Ltd. Japan), 0.1% BSA and 0.4M sucrose at 28 °C for 4 hr with gentle shaking on a gyratory shaker at 75 rpm. Protoplasts were filtered through a 90μm steel mesh screen and purified by centrifugation in Babcock bottles (Baxter Scientific Products) at (350 X g) for 10 min at room temperature. The protoplasts were recovered from the floating band and diluted in W5 solution [145 mM NaCl, 125 mM $CaCl_2 2H_2O$, 5mM KCl, 5mM glucose pH 5.6] (Negrutiu et al. 1987) and incubated at room temperature for 30 min. 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 (50 X g) for 10min and resuspended to a final concentration of 1.7 X 10^6 viable protoplasts per ml with MaMg solution [0.4 M mannitol, 15 mM $MgCl_2$, 3 mM morpholinoethanesulphonic acid (MES)-KOH pH 5.6] (Negrutiu et al., 1987). 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 mixture of 20 µg of CsCl purified pGA643 construct (no plasmid in negative control), and 50 µg sheared salmon sperm DNA was added to the protoplast suspension. The protoplast/plasmid DNA mixture was brought to a final concentration of 28% Polyethylene Glycol (PEG)-4000 with a solution containing 40% PEG 4000, 0.4 M mannitol, 100 mM $Ca(NO_3)_2 \cdot 4H_2O$, 10 mM N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-KOH pH 7.0 (Negrutiu et al., 1987). 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 as described by Damm et al. (1989). The Protoplasts were collected by centrifugation at 50 X g for 10 min at room temperature and the protoplast pellet was resuspended in 2.5 ml MS 0.2 mg/L 2,4-D, and 0.4 M mannitol to a final density of 2.0 X 10^5 protoplast/ml and transferred to 80 X 15 mm petri plates.

To examine expression of the barley lectin constructs, the transiently transformed NT protoplasts were incubated for 12 hours in the presence of 200 µCi ["Expre^{35}S^{35}S" ^{35}S protein labeling mixture, (NEN 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). After labeling, the protoplasts were separated from the culture media by centrifugation at (50 X g) for 10 min at room temperature. The protoplast pellet was resuspended in 200 µl extraction buffer [50 mM Tris-acetate pH 5.0, 100 mM NaCl, 0.6% triton X-100 and 0.6 mM dithiothreitol]. The lysate was cleared of insoluble debris by centrifugation at (16,000 X g) for 5 min at 4 °C

frozen in liquid N_2 and stored at -70 °C. The culture media (2.5 ml) was filtered to remove any remaining protoplasts as described in Wilkins et al. (1990). Proteins in the culture media were precipitated with ammonium sulfate at 70% saturation at 4 °C for 2 hours and then collected by centrifugation at 10,000 rpm for 10 min at 4 °C. The culture media protein pellet was resuspended in 200 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) 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], barley lectin was eluted with 150 µl 100 mM N-acetylglucosamine, and lyophilized. The radiolabeled barley lectin was analyzed by SDS-PAGE on 12.5% polyacrylamide gels and visualized by fluorography as detailed in Mansfield et al. (1988).

Suspension cell and Plant Transformation

The binary vector pGA643 construct containing *wt* or *ctpp* were mobilized to *Agrobacterium tumefaciens* LBA4404 as described in Wilkins et al. (1990). NT suspension cells were co-cultivated with agrobacteria harboring *wt* and *ctpp* pGA643 constructs according to An, (1985) and plated on MS 0.2mg/L 2,4-D agar supplemented with 500 mg/L carbenicillin and 150 mg/L kanamycin. After 3-4 weeks calli and were transferred to fresh selective media. Transformed calli expressing barley lectin were grown in liquid MS 0.2 mg/L 2,4-D media with 500 mg/L carbenicillin and 150 mg/L kanamycin on a gyratory shaker at 150 rpm at

28 °C. The *ctpp* transformed plants were obtained as described in Wilkins et al. (1990).

RNA Gel Blot Analysis

Total RNA was isolated from untransformed and transgenic tobacco suspension cells and plants as described (Nagy et al., 1988). 20 μg of total RNA from each sample was fractionated on 2% agarose gels containing 6% formaldehyde and blotted to nitrocellulose. The nitrocellulose blot was hybridized with ^{32}P random-primer labeled (Feinberg and Vogelstein, 1983) pBlc3 barley lectin cDNA insert (Lerner and Raikhel, 1989) and washed as previously described (Raikhel et al., 1988). Blots were air dried and exposed to XAR-5 film (Kodak) using intensifying screens at -70 °C. Autoradiograms were analyzed by scanning densitometry with a Beckman DU-64 spectrophotometer (Beckman Instruments).

Radiolabeling of Transgenic Suspension-Cells

For pulse-chase labeling experiments, 0.5ml suspension-cultured cells (per well) from 4 day old cultures were incubated in 24 well Falcon tissue culture plates in the presence of 85 μCi ^{35}S-met/cys (see above). 2 wells or a total of 1 ml of the 4 day suspension cell culture were labeled per time point. The cells were incubated at room temperature with gentle shaking on a gyrotary shaker at 75 rpm in the dark for 6 hours. After 6 hours, labeled proteins were chased by adding

unlabeled methionine and cysteine to a concentration of 5 mM and 2.5 mM respectively per well. At the appropriate time points labeled NT cell suspensions are pooled in 1.5 ml mircofuge tubes and the cells were separated from the media by centrifugation at 2,500 rpm for 1 min at 4 °C.

The culture media was transfered to another tube and centrifuged at (16,000 X g) for 10 min at 4 °C to remove any unpelleted cells and debris. Proteins in the culture media were concentrated as described above and stored at -70 °C. The cells were washed once with 500 µl MS 0.2mg/L 2,4-D pelleted by centrifugation at 2,500 rpm for 1 min at 4 °C. Cells were homogenized in 300 µl extraction buffer [50 mM Tris-acetate pH 5.0, 100 mM NaCl, 0.6% triton X-100 and 0.6 mM dithiothreitol]. To break the cells, the cell suspension was chilled slowly in liquid N_2 and the ice slurry was homogenized using a motor driven microfuge pestle (Kontes). The homogenate was centrifuged at (16,000 X g) for 10 min at 4 °C to remove debris and stored at -70 °C. Radiolabeled barley lectin was purified from the crude protein extracts and analyzed as described above.

Radiolabeling of Tobacco Leaf Protoplasts

Protoplast for labeling were prepared from fully expanded leaves of 4-6 week old axenic shoot cultures of W38-*wt* and W38-*ctpp*. Leaf protoplasts were prepared as described in Wilkins et al., (1990) with the exception that the enyzme mixture was dissolved in MS medium supplemented with 1.0 mg/l benzyladenine (BA), 0.1 mg/L napthaleneacetic acid (NAA) and 0.6 M mannitol. To remove broken protoplasts and undigested cells the protoplasts were pelleted at (50 X g) for 10

min resuspended in MS medium with 1.0 mg/l BA, 0.1 mg/L NAA and 0.6 M sucrose and centrifuged at (350 X g) for 10 min in Babcock bottles. The floating band of protoplasts was washed once and diluted in MS medium with 1.0 mg/l BA, 0.1 mg/L NAA and 0.6 M mannitol. Viable protoplasts were quantified as described above.

Pulse-labeling experiments of leaf protoplasts were performed as detailed in Wilkins et al. (1990), with the exception that the culture medium from each time point was recovered and analyzed as described above.

Vacuole Isolation from Labeled Tobacco Leaf Protoplasts

For vacuole isolation 1.2 X 10^6 protoplasts were incubated in a total of 3.0 mls of MS medium with 1.0 mg/l BA, 0.1 mg/L NAA and 0.6 M mannitol supplemented with 300 μCi ^{35}S-met/cys. Protoplasts were incubated in the dark at room temperature with gentle shaking (50 rpm on a gyratory shaker) for 12 hours. 2 X 10^5 labeled protoplasts were treated as described above (Radiolabeling of Tobacco Leaf Protoplasts) to confirm synthesis of radiolabeled barley lectin. The remaining 1 X 10^6 protoplasts were pooled and collected by centrifugation at (50 X g) for 5 min at 4 °C.

Vacuoles were isolated as described (Wilkins et al., 1990) and gently lysed by osmotic shock. Four volumes of 10 mM Hepes-KOH, pH 7.2 was added to the vacuole suspension and incubated at 4 °C for 30 min. Membranes and unbroken vacuoles were pelleted 30 min at (16,000 X g) at 4 °C. Soluble proteins were concentrated by precipitation with ammonium sulfate at 70% saturation at 4 °C for

at least 2 hours. Precipitated proteins were collected by centrifugation for 10 min at (16,000 X g) at 4 °C. The protein pellet was resuspended in 300 µl 10 mM Hepes-KOH pH 7.2. Activity of the vacuole-specific enzyme α-mannosidase was assayed as described by Boller and Kende (1979). ^{35}S-labeled barley lectin was purified and analyzed as described above.

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CHAPTER 4*

The barley lectin carboxyl-terminal propeptide is a vacuolar protein sorting determinant in plants

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ABSTRACT

We have previously shown that the 15 amino acid carboxyl-terminal propeptide of probarley lectin is necessary for the proper sorting of this protein to the plant vacuole. A mutant form of the protein lacking the carboxyl-terminal propeptide is secreted. To test whether the carboxyl-terminal propeptide is the vacuole sorting determinant of probarley lectin, we examined the processing and sorting of a series of fusion proteins, containing the secreted protein, cucumber chitinase and regions of probarley lectin, in transgenic tobacco. Pulse-labeling experiments demonstrated that the fusion proteins were properly translocated through the tobacco secretory system and that cucumber chitinase and cucumber chitinase fusion proteins lacking the carboxyl-terminal propeptide were secreted. The cucumber chitinase fusion protein containing the carboxyl-terminal propeptide was properly processed and sorted to the vacuole in transgenic tobacco as confirmed by organelle fractionation and electron microscopy immunocytochemistry. Therefore, the barley lectin carboxyl-terminal propeptide is both necessary and sufficient for protein sorting to the plant vacuole.

INTRODUCTION

The plant vacuole is a multifunctional organelle that is essential for the regulation and maintenance of plant cell growth and development (for a review, see Boller and Wiemken, 1986). Similar to the yeast vacuole and mammalian lysosome, the plant vacuole contains a large number of soluble hydrolytic enzymes. In addition, many other proteins, such as storage or plant defense proteins, may accumulate in the plant vacuole in response to specific developmental or environmental signals. The majority of these enzymes and proteins are delivered to the vacuole via the secretory system.

Proteins that enter the secretory system are either secreted or are specifically localized within distinct subcellular compartment such as the endoplasmic reticulum (ER), Golgi complex, plasma membrane, or the vacuole/lysosome. Entry into the secretory pathway as well as subsequent processing and sorting events require specific information provided by the protein (Blobel, 1980). Targeting and translocation of most plant, animal, and yeast secretory proteins from the cytosol into the ER is dependent on an amino-terminal hydrophobic signal sequence (see Chrispeels, 1991, for review). Within the ER lumen, many proteins are further modified and/or assembled to attain competence for transport through the secretory pathway (Chrispeels, 1991). In the absence of any additional sorting information, proteins exit the ER by "bulk flow" and are secreted by default (Rothman, 1987; Wieland et al., 1987; Denecke et al., 1990; Hunt and Chrispeels, 1991).

Proteins that are selectively localized within the secretory pathway require specific secondary sorting signal(s), consisting of either post-translational modifications and/or primary, secondary, or tertiary structural elements within the protein (Blobel, 1980; Rothman, 1987). A mannose 6-phosphate group on oligosaccharide side chains of many mammalian acid hydrolases serves as a lysosomal targeting signal (Kornfeld and Mellman, 1989). Within the trans-Golgi network, these modified sugars are recognized by mannose 6-phosphate receptors that mediate the sorting of these proteins to the lysosomes (von Figura and Hasilik, 1986). Ultimately though, the information for lysosomal sorting is contained within the structure of the lysosomal enzyme which specifies phosphorylation of the oligosaccharide side chain mannose residue (Kornfeld and Mellman, 1989).

Unlike the mannose-6-phosphate-dependent sorting of the lysosomal hydrolases, the transport and sorting of vacuolar proteins in plants and yeast are not dependent on protein glycosylation, but rather on direct recognition of elements within the polypeptide sequence or structure (Chrispeels, 1991). The targeting information for two yeast vacuolar proteins, carboxypeptidase Y (CPY) and proteinase A has been demonstrated to be contained within the amino-terminal propeptide of these proteins (Johnson et al., 1987; Valls et al., 1987; Klionsky et al., 1988). A detailed mutational analysis of the CPY propeptide identified a tetrapeptide Gln-Arg-Pro-Leu (QRPL) to be critical for sorting of the proprotein to the vacuole (Valls et al.,1990).

When the plant vacuolar protein phytohemagglutinin-L (PHA) was expressed

in yeast, it was properly sorted to the yeast vacuole (Tague and Chrispeels, 1987). Further analysis, identified a domain within the amino-terminal portion of PHA containing a tetrapeptide resembling the CPY sorting element Leu-Gln-Arg-Asp (LQRD), that is sufficient to redirect invertase to the yeast vacuole (Tague et al., 1990). However, the same PHA motif (LQRD) was not sufficient to target a reporter protein to vacuoles in Arabidopsis protoplasts (Chrispeels, 1991). These results suggest that the mechanisms of vacuole sorting in yeast and plants may not be entirely conserved.

We are interested in the molecular mechanisms regulating vacuolar sorting of the Gramineae lectins. Within the ER, the Gramineae lectin polypeptide is modified by the addition of a high mannose glycan to the carboxyl-terminal propeptide (CTPP) and is assembled to form an active N-acetylglucosamine-binding proprotein (Mansfield et al., 1988; Smith and Raikhel, 1989). During transport to or after deposition in the vacuole, the glycosylated CTPP is removed from the proprotein to yield the mature lectin. Previously, we have demonstrated the correct processing and accumulation of barley lectin (BL), in leaves and roots of transgenic tobacco plants (Wilkins et al., 1990). It was also shown that the glycan of the barley lectin proprotein (proBL) is not essential for correct processing and targeting of this protein to the vacuoles (Wilkins et al., 1990). Recently work from our laboratory has established that the CTPP is necessary for proper sorting of BL to vacuoles of tobacco (Bednarek et al., 1990). In this report, we present evidence that the barley lectin CTPP is both necessary and sufficient to redirect a secreted protein, cucumber chitinase, to plant vacuoles of tobacco.

RESULTS

Assembly of Cucumber Chitinase Gene Fusions

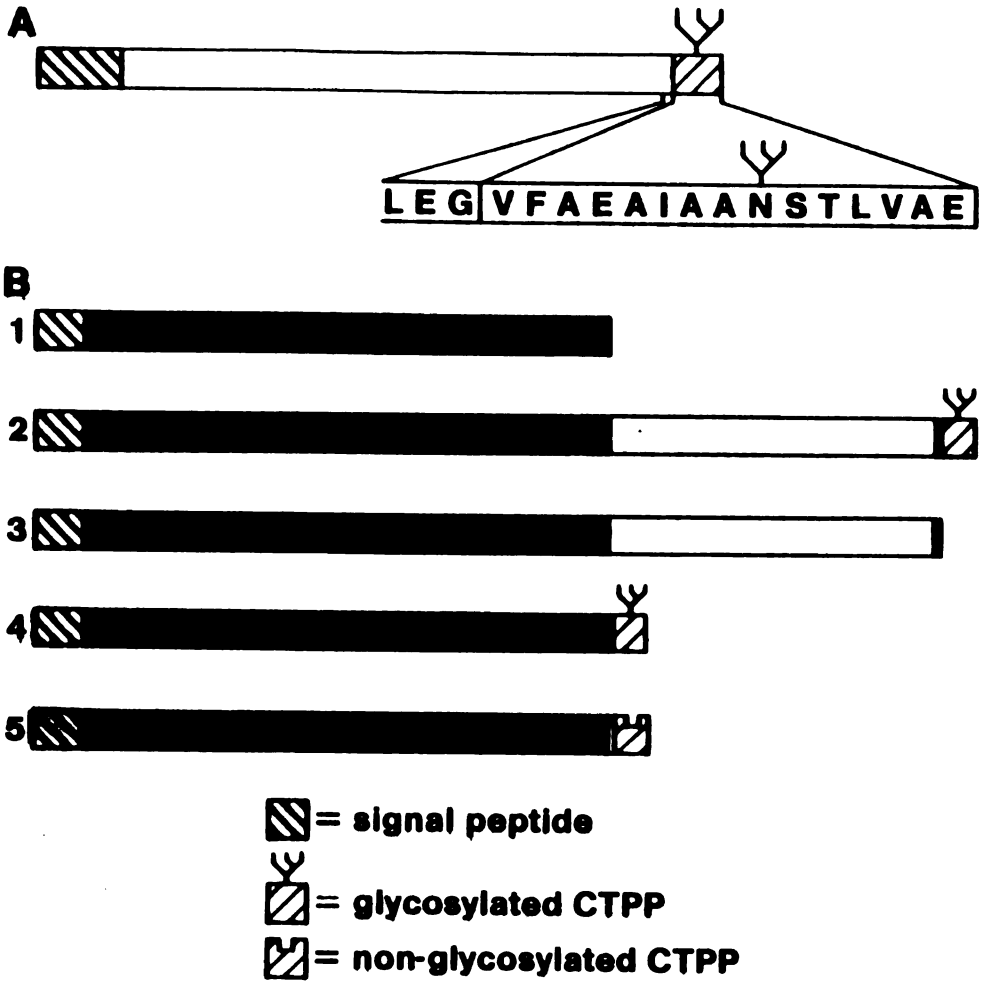
BL is a 36-kD homodimeric protein, which is localized in the vacuoles/protein bodies of embryonic and root cap cells of barley (Mishkind et al., 1983; Lerner and Raikhel, 1989). As shown in Figure 1A, each BL subunit is initially synthesized as a preproprotein composed of a 2.5-kD signal peptide, an 18-kD polypeptide, and a 1.5-kD CTPP. Within the ER, the proprotein is modified by the covalent addition of a high-mannose-type glycan to the CTPP, to form a 23-kD polypeptide, and dimerizes to form an active N-acetylglucosamine binding protein. During transport to or concomitant with deposition of the protein in the vacuole, the glycosylated CTPPs are cleaved to yield the dimer consisting of two 18-kD subunits.

We have previously demonstrated that BL is correctly processed and targeted in transgenic tobacco cells (Wilkins et al., 1990). Deletion of the 15 amino acid CTPP resulted in secretion of BL indicating that the CTPP is necessary for the proper sorting of this protein to the vacuoles of plant cells (Bednarek et al., 1990). To examine whether the CTPP is necessary and sufficient to redirect a reporter protein to plant vacuoles, a chimeric gene containing the cDNA encoding cucumber chitinase (Cuc Chit) (Metraux et al., 1989) was fused with the region of the BL cDNA encoding the CTPP (Lerner and Raikhel, 1989) (Figure 1, B4). In addition, two gene fusions were constructed to determine whether there are any additional topogenic signals within the mature barley lectin 18 kD polypeptide sequence necessary for vacuolar protein sorting (Figure 1, B2, and B3).

Figure 1. Schematic Representation of proBL/Cuc Chit Fusion Proteins

(**A**). The preproprotein of barley lectin consists of a signal sequence (box with dark hatched lines), a mature 18 kD subunit (open box), and the CTPP (box with light hatched lines). The insert represents the 15-amino acid CTPP propeptide (lightly shaded box), Gly (G) is the last amino acid from the carboxyl-terminus of the mature BL preceding the CTPP. Leu (L) and Glu (E) (open box) were added by introduction of a XhoI restriction site (see Methods).

(**B**). (1) The preprotein of Cuc Chit contains a signal sequence (shaded box) and the mature 28-kD polypeptide (solid black box). (2) Cuc Chit is fused with the 23-kD glycosylated BL proprotein (Cuc Chit-proBL). (3) Cuc Chit is fused with the mature 18-kD BL subunit (Cuc Chit-BL). (4) Cuc Chit is fused with the glycosylated CTPP (Cuc Chit-CTPP). (5) Cuc Chit-CTPP fusion protein that has been modified by site-directed mutagenesis to prevent core glycosylation of the CTPP (Cuc Chit-CTPP[gly$^-$]).


A
LEG
VFAEAIAANSTLVAE
B
1
2
3
4
5
= signal peptide
= glycosylated CTPP
= non-glycosylated CTPP

Cuc Chit is a protease-resistant 28-kD protein that is secreted into the intercellular space of cucumber plant in response to viral or pathogen infection (Metraux et al., 1989). No significant homology is found in a comparison of the DNA and deduced amino acid sequences of Cuc Chit and the intracellular basic chitinase isoforms from tobacco and bean (Metraux et al., 1989). In addition, polyclonal anti-Cuc Chit antisera does not cross react with the chitinases from tobacco (J. Ryals, personal communication). Endonuclease restriction sites were introduced by site-directed mutagenesis (see Methods) into cDNA genes encoding Cuc Chit and proBL to facilitate subcloning of proBL restriction fragments onto the 3' end of the Cuc Chit open reading frame. Figure 1B, is a schematic representation of the proteins encoded by the proBL/CucChit restriction fragment gene fusions. Three Cuc Chit gene fusions were constructed containing the following sequences: (1) the region encoding the barley lectin proprotein (Figure 1, B2), (2) the region encoding only the mature 18-kD subunit (Figure 1, B3), and (3) the CTPP coding region (Figure 1, B4). Although it has been demonstrated that the last carboxyl-terminal amino acid of the mature lectin subunit is a glycine residue (Gly^{171}) (Wright et al., 1984), the exact site within proBL at which the CTPP is cleaved has yet to be defined. For this reason, we have engineered the 3' end of the Cuc Chit open reading frame to mimic the last two carboxyl-terminal amino acids of the mature 18-kD barley lectin subunit. The Cuc Chit-CTPP fusion gene was assembled (see Methods) such that the carboxyl-terminal amino acids of Cuc Chit preceding the CTPP, were an acidic amino acid (Glu) followed by a glycine residue.

Cuc Chit gene fusions were subcloned into the plant expression vector pGA643 (An et al., 1988) under transcriptional control of the cauliflower mosaic virus 35S promoter. The resulting constructs were transiently expressed in tobacco suspension-cell protoplasts or stably transformed as described (Wilkins et al., 1990; Bednarek et al., 1990) into tobacco cells and plants via Agrobacterium.

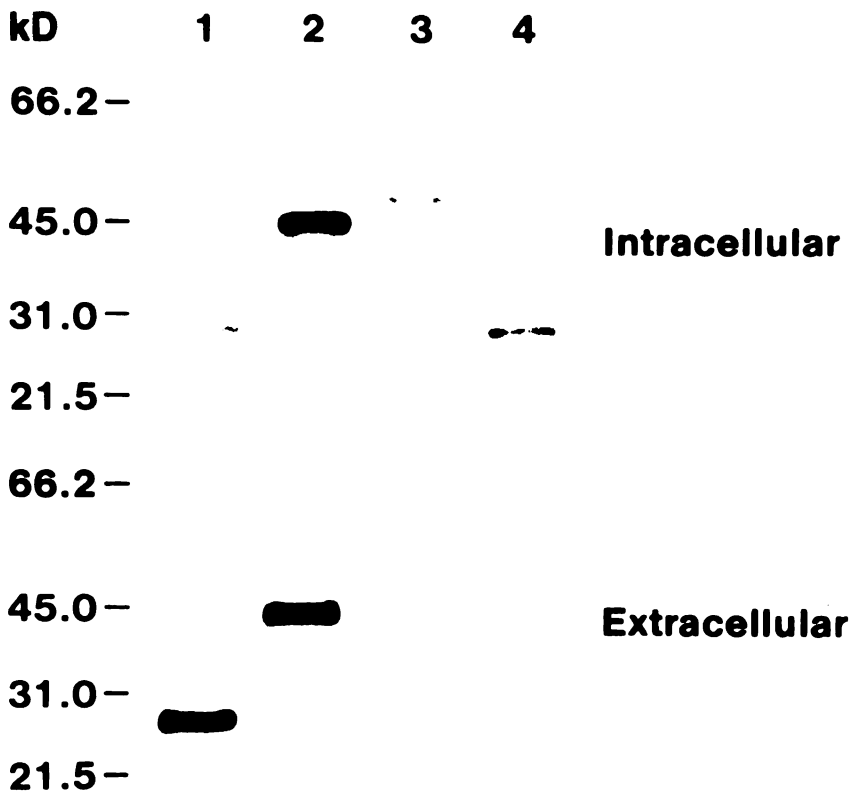
Analysis of Cuc Chit Gene Fusions in Transformed Tobacco Cells

To facilitate a rapid analysis of the proBL/Cuc Chit fusion proteins, Cuc Chit/pGA643 constructs were introduced into tobacco suspension-cell protoplasts by polyethylene glycol mediated DNA uptake (Bednarek et al., 1990), and the protoplasts were labeled

in the presence of a mixture of ^{35}S-labeled methionine and cysteine (^{35}S-Met/Cys) for 14 hr. Cuc Chit and Cuc Chit fusion proteins were purified from protein extracts of the radiolabeled protoplasts and incubation media by immunoprecipitation with polyclonal antisera directed against Cuc Chit and analyzed by SDS-PAGE and fluorography. As shown in Figure 2, Cuc Chit and Cuc Chit-BL were synthesized as single polypeptides of M_r 28,000 and M_r 46,000, respectively, and secreted from the protoplasts into the incubation media (Figure 2, lanes 1, 2), indicating that these proteins were properly translocated into the tobacco secretory system and secreted. The labeled polypeptide with an M_r of 46,000 was completely secreted from the tobacco protoplast during a 10 hr chase with unlabeled methionine and cysteine (Met/Cys) and accumulated in the media

Figure 2. Analysis of Transiently Expressed Cuc Chit Fusion Proteins In Tobacco Protoplasts.

Cuc Chit/pGA643 constructs were introduced into tobacco protoplasts by PEG-mediated DNA uptake. Immunopurified proteins from the intracellular and extracellular fractions of pulse labeled tobacco protoplasts expressing, Cuc Chit (lane 1), Cuc Chit-BL (lane 2), Cuc Chit-proBL (lane 3), and Cuc Chit-CTPP (lane 4) were electrophoresed on 12.5% SDS-polyacrylamide gels and visualized by fluorography. The migration of molecular mass markers (kD) is represented on the left.


kD
1
2
3
4
66.2
45.0
31.0
21.5
66.2
45.0
31.0
21.5
Intracellular
Extracellular

(data not shown). In tobacco protoplasts transformed with Cuc Chit-proBL, two polypeptides of M_r 51,000 (Figure 1, B2) and M_r 46,000 were detected intracellularly (Figure 2, lane 3). These results imply that the Cuc Chit-proBL fusion protein was modified by glycosylation (M_r 51,000) and subsequently processed by removal of the glycopropeptide to the polypeptide with an M_r of 46,000. Likewise, in tobacco protoplasts transformed with Cuc Chit-CTPP, two polypeptides of M_r 33,000 (corresponding to the predicted molecular mass of glycosylated Cuc Chit-CTPP) and M_r 28,000 were detected intracellularly (Figure 2, lane 4). The presence of the intracellular polypeptide with an M_r of 28,000 suggests that Cuc Chit-CTPP was properly sorted and processed in tobacco cells. In addition, a single polypeptide (M_r 33,000) corresponding to Cuc Chit-CTPP proprotein was detected in the incubation media of tobacco protoplasts transformed with Cuc Chit-CTPP (Figure 2, lane 4). A very low level of a protein (M_r 51,000) was secreted from tobacco protoplasts expressing Cuc Chit-proBL. The secreted radiolabeled polypeptide with an M_r of 51,000 was discernible only after a very long exposure (> 4 weeks) of the fluorogram shown in Figure 2, (data not shown).

The levels of processed and secreted Cuc Chit-proBL and Cuc Chit-CTPP fusion proteins were compared by densitometric analysis of the fluorogram in Figure 2. The majority of the Cuc Chit-proBL and Cuc Chit-CTPP fusion proteins (approximately 95% and 75% respectively), were processed and retained intracellularly.

To further analyze the synthesis and processing of Cuc Chit fusion proteins, protoplasts from stably transformed Cuc Chit and Cuc Chit-CTPP transgenic

tobacco plants were pulse-labeled for 2.5 hr with ^{35}S-Met/Cys. Labeled proteins were chased with Met/Cys for an additional 8 hr. Intracellular and extracellular proteins were purified by immunoprecipitation with anti-Cuc Chit antisera and analyzed by SDS-PAGE and fluorography as shown in Figure 3. As expected for a secreted protein, the level of Cuc Chit (M_r 28,000) decreased intracellularly and correspondingly increased in the incubation media over the course of the chase (Figure 3A). At the start of the chase a polypeptide with a M_r of 33,000 was detected in Cuc Chit-CTPP protoplasts and at a low level in the incubation media (Figure 3B, 0 hr). During the 8 hr chase, the polypeptide with an M_r of 33,000 became almost undetectable in the protoplasts and was accompanied by a corresponding increase in the level of a polypeptide with an M_r of 28,000. The level of unprocessed Cuc Chit-CTPP (M_r 33,000) in the media increased slightly during the chase time course. These results imply that the Cuc Chit-CTPP proprotein (M_r 33,000) was processed to a polypeptide with an M_r of 28,000 and retained intracellularly. The rate of Cuc Chit and Cuc Chit-CTPP secretion and processing were quantitated by densitometric analysis of SDS-PAGE fluorogram (Figure 3). At room temperature Cuc Chit was secreted from tobacco leaf protoplast with a half-life ($t_{1/2}$) of approximately 1.5 hr. Processing of the Cuc Chit-CTPP fusion protein occurred with a $t_{1/2}$ of 2.1 hr.

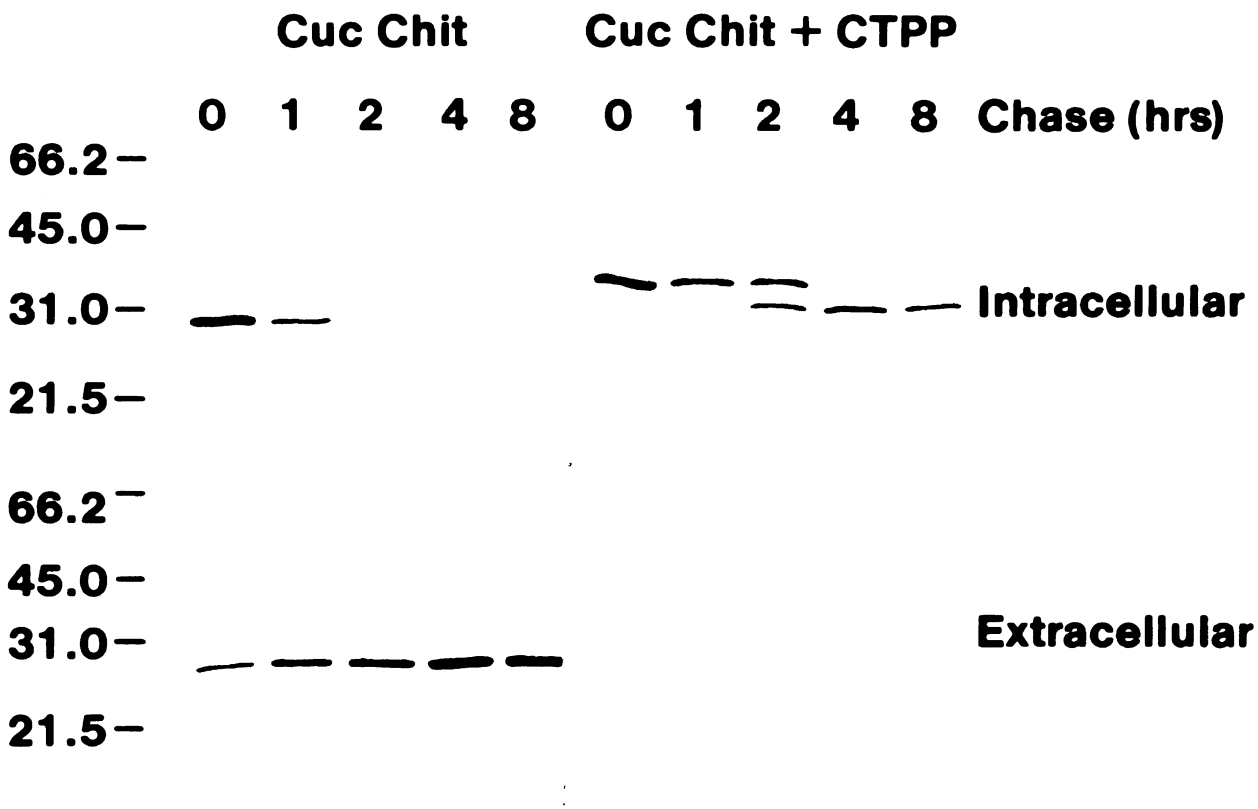
Subcellular Localization of Cuc Chit and Cuc Chit-CTPP Fusion Proteins

We have previously shown that the 23-kD proprotein and the mature 18-kD subunit of BL are localized in the microsomal fraction and vacuoles of transgenic tobacco

Figure 3. Pulse-Chase Labeling Experiments of Tobacco Protoplasts Expressing Cuc Chit and Cuc Chit-CTPP Fusion Proteins.

A. Immunopurified intracellular and extracellular proteins from tobacco protoplasts expressing Cuc Chit.

B. Immunopurified intracellular and extracellular proteins from tobacco protoplasts expressing Cuc Chit-CTPP. Protoplasts were pulse labeled for 2.5 hr and chased for 8 hr. Protein extracts were prepared from the protoplasts and incubation media at specified intervals during the chase as indicated. Radiolabeled proteins were immunoprecipitated with anti-Cuc Chit antisera and analyzed by SDS-PAGE and fluorography. Molecular mass markers (kD) are indicated on the left.


Cuc Chit
Cuc Chit + CTPP
0 1 2 4 8 0 1 2 4 8 Chase (hrs)
66.2
45.0
31.0
21.5
Intracellular
66.2
45.0
31.0
21.5
Extracellular

cells, respectively (Wilkins et al., 1990). The subcellular localization of Cuc Chit and Cuc Chit-CTPP were examined by organelle isolation as shown in Figure 4 and by electron microscopic immunocytochemistry as shown in Figure 5. Protoplasts for vacuole isolation were prepared from the same transgenic plants used in the pulse/chase experiments (Figure 3) to insure similar levels of Cuc Chit and Cuc Chit-CTPP fusion protein expression and vacuoles were isolated as described in Methods. Activities of enzymes specific for the cytosol (glucose-6-phosphate dehydrogenase, EC 1.1.1.49)(Simcox et al., 1977), the ER (NADH cytochrome c reductase, EC 1.6.99.3) (Lord, 1983), and the vacuole (α-mannosidase, EC 3.2.1.24) (Boller and Kende, 1979) were compared in crude protoplast and vacuole lysates. Vacuole fractions from Cuc Chit and Cuc Chit-CTPP plants contained $\leq$ 10% NADH cytochrome c reductase and $\leq$ 5% glucose-6-phosphate dehydrogenase, relative to total protoplast associated activity. The subcellular distribution of Cuc Chit and Cuc Chit-CTPP proteins was examined by immunoblot analysis of the protoplast and vacuole lysates using Cuc Chit polyclonal sera. Gels were loaded such that each lane contained the same amount of total vacuolar protein, with respect to α-mannosidase activity. The processed form of Cuc Chit-CTPP proprotein (M, 28,000) was detected only in the vacuole fraction from Cuc Chit-CTPP protoplasts, indicating that the CTPP is sufficient for redirection of Cuc Chit to the vacuoles of plants (Figure 4, lane 4).

Localization of Cuc Chit in transgenic tobacco plants was also analyzed by electron microscopic immunocytochemistry. Thin sections of transgenic tobacco leaves expressing Cuc Chit and Cuc Chit-CTPP were treated with Cuc Chit

Figure 4. Localization of the processed form of the Cuc Chit-CTPP fusion protein in the vacuoles of Cuc Chit-CTPP transgenic tobacco protoplasts.

Total protein from protoplasts and isolated vacuoles were separated by electrophoresis on a 12.5% SDS-polyacrylamide gel, and electroblotted onto Immobilon-P membrane. Immunodetection of Cuc Chit was performed with anti-Cuc Chit antisera as described in Methods. Equal amounts of soluble vacuole proteins in the protoplast and vacuole fractions, relative to α-mannosidase activity, were loaded per lane. The sizes of molecular mass standards (kD) are shown on the left.

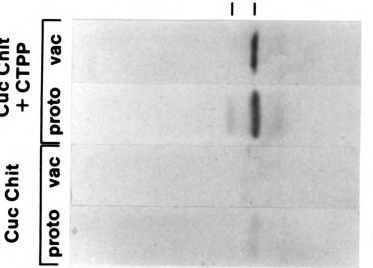
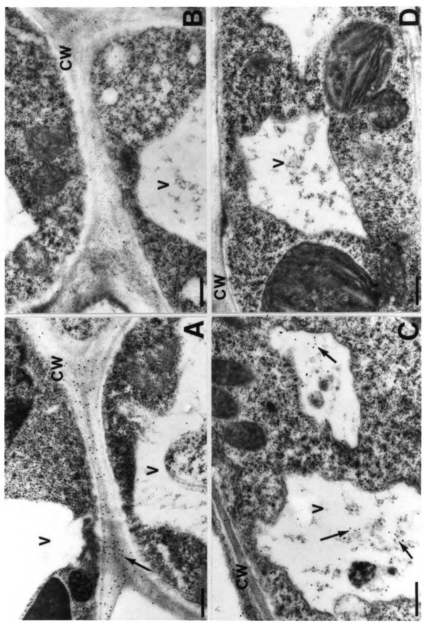

Cuc Chit
Cuc Chit + CTPP
kD
proto
vac
proto
vac
66.2
45.0
31.0
21.5
Cuc Chit + CTPP
Cuc Chit
1
2
3
4

Figure 5. Immunocytochemical Localization of Cuc Chit and Cuc Chit-CTPP Fusion in Transgenic Tobacco Cells.

(A) and (C) Thin sections of transgenic tobacco leaves expressing Cuc Chit (A) and Cuc Chit-CTPP (C) treated with anti-Cuc Chit antisera.

(B) and (D) Thin sections of transgenic tobacco leaves expressing Cuc Chit (B) and Cuc Chit-CTPP (D) treated with nonimmune sera.

Antibody binding was visualized by protein A-gold (15 nm). Gold label (arrow) is found exclusively in the cell wall of tobacco plants transformed with Cuc Chit transcript (panel A) and within the vacuoles of transgenic Cuc Chit-CTPP tobacco plants (panel C). Bars = 0.5 μm. The abbreviations used are: CW, cell wall; V, vacuole.


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

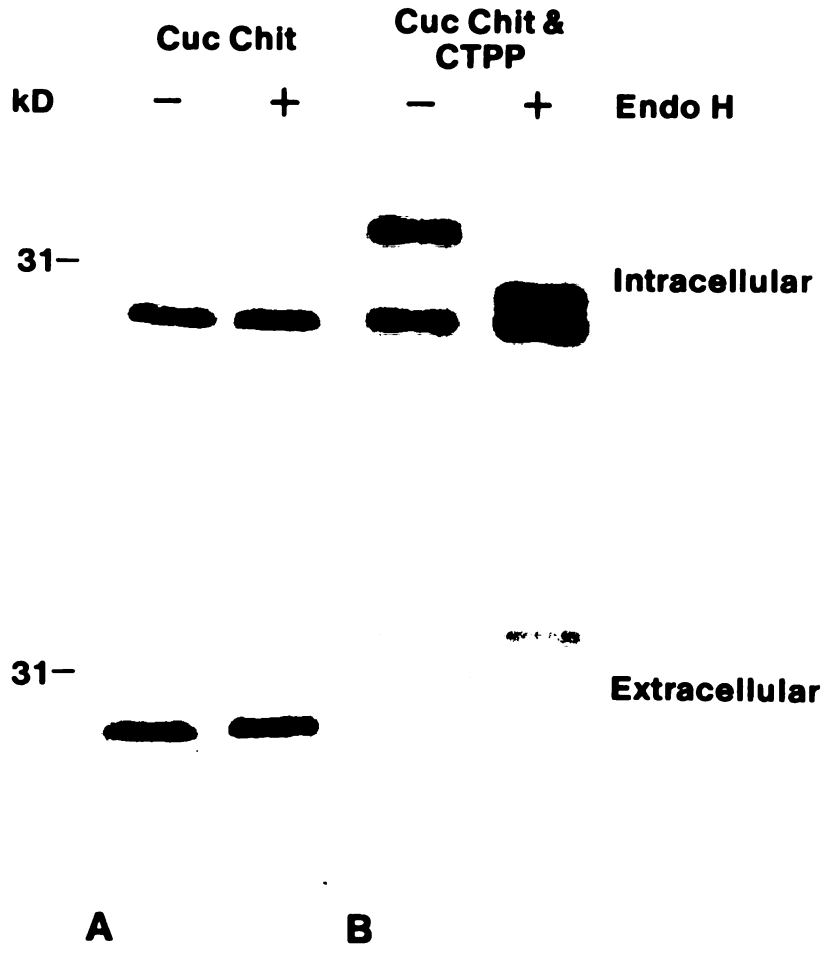
antiserum. Antibody binding was visualized with 15-nm-diameter colloidal gold linked to protein A. Cuc Chit was localized in the cell wall and middle lamella of tobacco cells expressing Cuc Chit (Figure 5A), whereas colloidal gold labeling was readily discernible in the vacuoles of tobacco cells expressing Cuc Chit-CTPP (Figure 5C). A very low level of labeling was also detected in the cell wall of these cells. No specific labeling was detected in parallel experiments using non-immune sera as the primary antibody (Figure 5B and 5D).

Glycosylation of Cuc Chit-CTPP Fusion Protein

In barley embryos and transgenic tobacco, proBL is modified by the addition of a high mannose oligosaccharide with a molecular mass of approximately 2-kD to the CTPP (Lerner and Raikhel, 1989; Wilkins et al., 1990). The mobility of Cuc Chit-CTPP proprotein (M_r 33,000) on SDS-polyacrylamide gels suggested that the CTPP was similarly modified in the fusion protein. To examine whether the CTPP of Cuc Chit-CTPP was glycosylated, immunoprecipitated proteins from radiolabeled Cuc Chit-CTPP protoplasts and incubation media were digested with endo-β-N-acetylglucosamine H (endo H) an enzyme which specifically cleaves high-mannose oligosaccharide side chains. The majority of intracellular Cuc Chit-CTPP (M_r 33,000) was converted to a protein with an M_r of 30,000 by treatment with endo H as shown in Figure 6B. Interestingly, the extracellular polypeptide (M_r 33,000) was insensitive to digestion with endo H (Figure 6B). We were unable to establish whether the endo H-resistant oligosaccharide side chain of Cuc Chit-CTPP proprotein was the result of modification of the high-mannose glycan to a

Figure 6. Endo H Digestion of Radiolabeled Cuc Chit and Cuc Chit-CTPP Fusion Protein.

Radiolabeled proteins were immunopurified from the intracellular and extracellular fractions of tobacco protoplasts expressing Cuc Chit and Cuc Chit-CTPP. Duplicate samples were incubated at 37° C for 18 hr in the absence or presence of endo H prior to analysis by SDS-PAGE and fluorography.


Cuc Chit
Cuc Chit &
CTPP
kD
–
+
–
+
Endo H
31–
Intracellular
31–
Extracellular
A
B

complex type (data not shown). ^{35}S-labeled proBL was treated with endo H as a positive control for endo H activity. As previously described (Wilkins et al., 1990), proBL (molecular mass 23-kD) was deglycosylated by endo H treatment to a 21-kD polypeptide (data not shown).

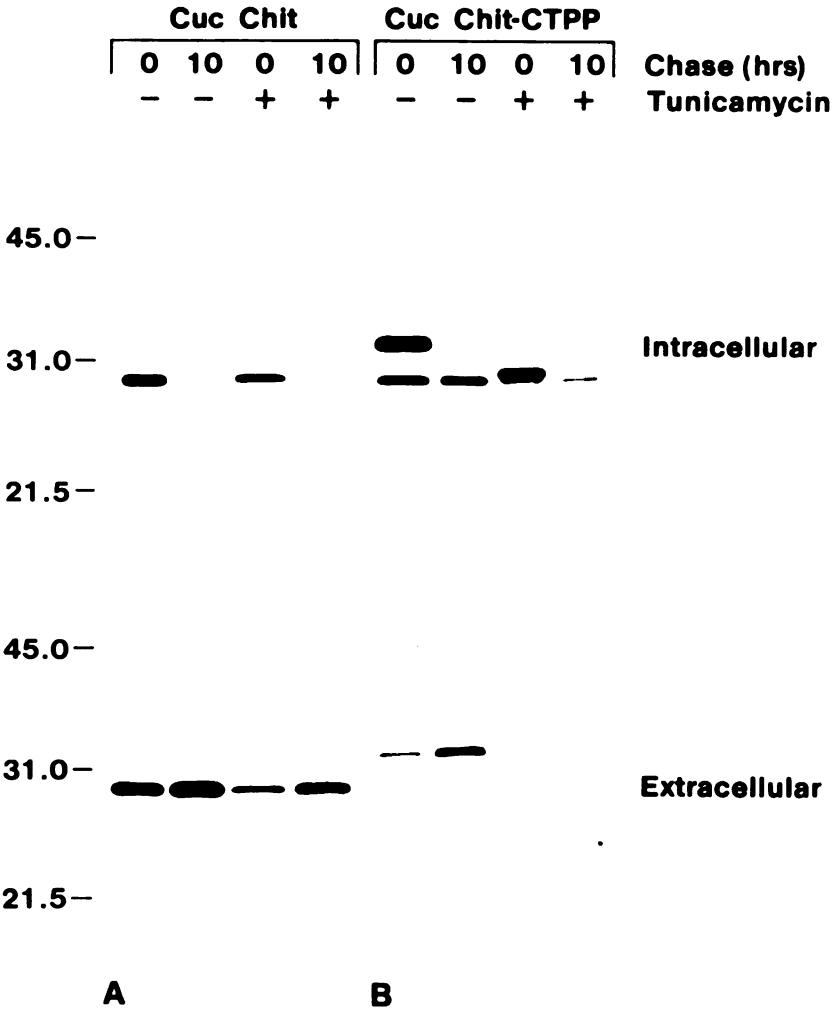
We have examined the possibility that secretion of Cuc Chit-CTPP (M_r 33,000) was resulted from the presence of the endo H-resistant glycan on the proprotein. Protoplasts expressing Cuc Chit-CTPP were labeled for 4 hr with ^{35}S-Met/Cys in the presence of 25 μM tunicamycin to block N-linked glycosylation. Labeled proteins were chased for an additional 10 hr with excess unlabeled Met/Cys as shown in Figure 7. Similar experiments were performed with a mutant form of Cuc Chit-CTPP in which the CTPP glycosylation site (Asn-Ser-Thr) was altered by site directed mutagenesis (Gly-Ser-Thr) to prevent attachment of the N-linked glycan (see Figure 1, B5, for a schematic representation of the Cuc Chit-CTPP[Gly$^-$] fusion protein) (data not shown). Levels of Cuc Chit were quantitated in intracellular and extracellular fractions from tunicamycin-treated Cuc Chit-CTPP protoplasts and tobacco protoplasts expressing Cuc Chit-CTPP[gly$^-$] as described above. Inhibition of Cuc Chit-CTPP glycosylation by either method only slightly increased the level (80 to 85%) of the Cuc Chit-CTPP proprotein which was processed and retained intracellularly.

Stability of Cuc Chit in the Vacuole and Media

To examine the stability of Cuc Chit and Cuc Chit-CTPP protein in the vacuole and media, protoplast were pulse-labeled for 2.5 hr with ^{35}S-Met/Cys and radiolabeled

Figure 7. The Effect of Core Glycosylation Inhibition on Sorting of the Cuc Chit-CTPP Proprotein to the Vacuole.

Protoplasts expressing Cuc Chit and Cuc Chit-CTPP were labeled in the presence or absence of tunicamycin and ^{35}S-labeled proteins were chased for 10 hr with excess Met/Cys. Proteins were immunopurified with anti-Cuc Chit antisera from protoplasts and incubation media, and analyzed by SDS-PAGE and fluorography. The migration of molecular mass standards (kD) is shown on the left.


Cuc Chit
Cuc Chit-CTPP
0 10 0 10 0 10 0 10
Chase (hrs)
- - + + - - + +
Tunicamycin
45.0
31.0
21.5
Intracellular
45.0
31.0
21.5
Extracellular
A
B

proteins were chased with unlabeled Met/Cys for an additional 10 hr to deplete the intracellular pool of ^{35}S-labeled Cuc Chit and Cuc Chit-CTPP proprotein. Intracellular and extracellular proteins were purified by immunoprecipitation at 2 to 4 hr intervals during an additional 20 hr chase period. The rate of Cuc Chit turnover in the vacuole and media was quantitated by densitometry. The polypeptide with an M_r of 28,000 was degraded with a $t_{1/2}$ of 5 hr in the vacuole. No degradation of either Cuc Chit (M_r 28,000) or Cuc Chit-CTPP (M_r 33,000) in the media was observed.

DISCUSSION

Sorting of vacuolar and lysosomal proteins from other secretory proteins requires specific targeting information contained within the molecular structure of these polypeptides. Chimeric proteins containing a secreted protein and various regions of a vacuolar protein have been used to characterize vacuolar targeting information in yeast (Johnson et al., 1987; Klionsky et al., 1988). Using a similar approach, we have demonstrated that the vacuolar sorting determinant of the plant protein BL, is contained within a 15 amino acid CTPP. BL is initially synthesized as a glycosylated proprotein and is subsequently processed prior to or concomitant with depqsition of the protein in the vacuole, by removal of the glycosylated CTPP. Similarly, both Cuc Chit-CTPP and Cuc Chit-proBL fusion proteins were initially synthesized as proproteins and processed to their mature form by the removal of

the CTPP, intracellularly. We have further demonstrated by organelle purification and electron microscopy immunocytochemical localization, that the M, 28,000 processed form of Cuc Chit-CTPP is localized in the vacuoles of tobacco cells expressing Cuc Chit-CTPP. Thus, the proBL CTPP was sufficient to redirect a secreted protein, cucumber chitinase, to the plant vacuole.

We have analyzed the role of the 18-kD subunit of BL on the sorting of Cuc Chit-BL and Cuc Chit-proBL fusion proteins. The Cuc Chit-proBL fusion protein was processed by removal of the CTPP and the mature protein was retained intracellularly, whereas the Cuc Chit-BL fusion protein lacking the CTPP was efficiently secreted from the cell. Similarly, a mutant form of BL lacking the CTPP was secreted from transgenic tobacco protoplasts (Bednarek et al., 1990). Together, these results suggest that within the 18-kD subunit of BL there are no additional targeting elements sufficient for sorting of the protein to the vacuole.

Sorting Efficiency

Redirection of cucumber chitinase by the CTPP to the vacuole was not complete. We found that 70 to 75% of total radiolabeled Cuc Chit was localized in the vacuole and the remaining Cuc Chit-CTPP proprotein was secreted into the incubation media. In contrast to the mixed distribution of Cuc Chit-CTPP, the Cuc Chit-proBL fusion protein was efficiently retained intracellularly (95%, Figure 2, lane 3). The additional 18-kD BL subunit may present the CTPP in a more favorable structural context for sorting. Insertion of a "random spacer" peptide preceding the CTPP in the Cuc Chit-CTPP fusion protein may, likewise, facilitate efficient

vacuolar sorting.

Targeting element(s) within a fusion protein may not be presented in the proper secondary and/or tertiary structural context and result in the complete or partial secretion of chimeric protein. Johnson et al. (1987) determined that the first 30 amino acids of the yeast vacuolar CPY proprotein efficiently retained the secreted protein invertase, intracellularly, whereas a 10-amino acid region of the CPY propeptide was only effective at retaining 45% of the invertase fusion protein. Valls et al. (1990) suggest that fusion or deletions near this region containing the tetrapeptide QRPL, which is critical for CPY sorting, may interfere with the structural context in which the signal is presented and result in missorting of the CPY or the CPY-invertase fusion protein.

Evidence that the CTPP is presented in a different structural context in the Cuc Chit-CTPP fusion protein than is found in proBL is suggested by our observation that the intracellular and extracellular pools of Cuc Chit-CTPP proprotein differ in their sensitivity to endo H. The proBL CTPP is modified by a high-mannose oligosaccharide side chain as confirmed by enzymatic deglycosylation with endo H (Lerner and Raikhel, 1989; Smith and Raikhel, 1989). In contrast, only 70 to 75% of intracellular Cuc Chit-CTPP proprotein oligosaccharide side chain was cleaved by endo H, and the extracellular form of Cuc Chit-CTPP was completely insensitive to this endoglycosidase. Retention of the Cuc Chit-CTPP fusion protein was only slightly enhanced when core glycosylation of the CTPP was blocked. Therefore it is likely that the presence of the endo H-resistant glycan does not affect sorting. Instead, factors such as the

conformation of the Cuc Chit-CTPP fusion protein alter or mask the propeptide targeting information resulting in inefficient sorting of the protein in addition to rendering the CTPP glycan endo H resistant.

Analysis and Comparison of Plant Vacuolar Sorting Determinants

In addition to the Gramineae lectins, other soluble vacuolar proteins have been identified that are processed by removal of a carboxyl-terminal propeptide (see Chrispeels, 1991 for review). Similar to BL, the carboxyl-terminal extension of the basic isoform of tobacco chitinase is also necessary for sorting and sufficient to redirect a cucumber chitinase fusion protein to the vacuole (Neuhaus et al., in press). The primary sequences of the proBL CTPP and the basic tobacco chitinase isoform carboxyl-terminal extension are not homologous.

The basic and acidic isoforms of β-1,3-glucanases and chitinases from tobacco have been shown to be localized intracellularly and extracellularly, respectively (see Chrispeels, 1991 for review). A comparison of the deduced amino acid sequences of the acidic and basic β-1,3-glucanase and chitinase isoforms reveals that the vacuolar isoforms contain additional carboxyl-terminal extensions not found on the extracellular isoforms (Linthorst et al., 1990; Neale., 1990). Similar to BL, tobacco β-1,3-glucanase is initially synthesized as a glycosylated precursor and processed to the mature protein by removal of the glycosylated carboxyl-terminal propeptide (Shinshi et al., 1988; Van den Bulcke et al., 1989); however, it remains to be determined whether the propeptide contains any sorting information.

In addition to carboxyl-terminal extensions, many plant and yeast vacuolar proteins are synthesized as precursors with an amino-terminal propeptide that is proteolytically removed just before or upon arrival in the vacuole. The amino-terminal propeptides of both CPY and the yeast vacuolar protein proteinase A contain elements sufficient for sorting to the vacuole (Johnson et al., 1987; Valls et al., 1987; Valls et al., 1990; Klionsky et al., 1988). An amino-terminal propeptide from the sweet potato storage protein, sporamin, also contains a region necessary for vacuolar protein sorting in plants (Matsuoka and Nakamura, 1991). Deletion of this region led to secretion of the sporamin by transgenic tobacco cells, while prosporamin was processed and deposited within the vacuoles. However, to date, this region has not been demonstrated to be sufficient for sorting to the vacuole.

It is tempting to speculate that because a propeptide region is accessible to proteolytic processing, it would also present a sorting determinant in an accessible or favorable context. To date, no common consensus sequences or structural elements that function as vacuole localization signals in these amino and carboxyl-terminal propeptides have been identified. In addition, many plant vacuolar proteins are not synthesized as proproteins. Investigations into the mechanisms of sorting of PHA (Tague et al., 1990) and the 11S globulin legumin (Saalbach et al., 1991) have found multiple regions of targeting information within these proteins. These results suggests there may be multiple independent mechanisms for vacuolar protein sorting.

Conclusions

We have previously shown that deletion of the CTPP results in secretion of BL. Together with the data presented here, these results indicate that the BL CTPP is both necessary and sufficient for vacuole protein sorting. Additional studies are in progress to define and characterize the essential features of the CTPP for vacuole protein targeting and to use this data to elucidate the mechanisms which recognize these signals and mediate the sorting process.

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.

Construction of Cuc Chit Gene Fusions

pSCU1 contained a cucumber chitinase gene (Metraux et al., 1989) in which the putative Cuc Chit signal sequence coding region (amino acids 1 to 26) had been replaced with the signal peptide DNA sequence from the basic tobacco chitinase (amino acids 1 to 26) (Shinshi et al., 1987; 1990). The restriction fragment containing the Cuc Chit insert from pSCU1 was subcloned into pUC118 (Viera and Messing,, 1987). SalI and XbaI restriction sites were inserted in the 5' untranslated region of Cuc Chit by site-directed mutagenesis (Kunkel et al., 1987). An additional XhoI site was created by site-directed mutagenesis preceding the codon

for Gly^{289} of the Cuc Chit deduced amino acid sequence (Metraux et al., 1989). This construct will be called Cuc Chit (Figure 1, B1).

Two separate oligonucleotides were used to insert XhoI restriction sites by site-directed mutagenesis into the BL cDNA (Lerner and Raikhel, 1989) in pUC118 (Wilkins et al., 1990) . Three BL cDNA mutants were constructed containing the following XhoI site(s): (1) BL1 had a single XhoI site that preceded the codon for Gln^{27}, the first amino acid of the mature 18-kD subunit of BL; (2) BL2 had a single XhoI site that preceded the codon for gly^{197}; (3) BL3 was a double BL cDNA mutant containing both XhoI sites presented in BL1 and BL2.

The Cuc Chit gene fusions were constructed as follows: Cuc Chit-proBL was constructed by cloning an SalI-XhoI restriction fragment containing the Cuc Chit coding region into the SalI-XhoI restriction sites of BL1 in pUC118; Cuc Chit-BL was constructed by cloning the XhoI restriction fragment from BL3 into the XhoI restriction site of Cuc Chit; Cuc Chit-CTPP was constructed by cloning the SalI-XhoI restriction fragment of Cuc Chit into the SalI-XhoI restriction sites of BL2. The Cuc Chit-CTPP[Gly^-] gene fusion was constructed by altering the CTPP N-linked glycosylation site within the Cuc Chit-CTPP gene fusion as described previously (Wilkins et al., 1990). All mutations and constructs were checked and confirmed by ^{35}S dideoxy sequencing (Sanger et al., 1977). XbaI restriction fragments containing Cuc Chit and Cuc Chit gene fusions were subcloned into the XbaI site of the plant expression vector pGA643 (An et al., 1988).

Transient Gene Expression in Tobacco Suspension Protoplasts

Cuc Chit and Cuc Chit gene fusions were introduced into tobacco protoplasts as described previously (Bednarek et al., 1990), with the exception that the transiently transformed protoplasts were resuspended to a final density of 5.0 X 10^5 protoplasts per ml in 1.0 ml of liquid Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 0.2 mg/L 2,4-D and 0.4 M betaine monohydrate.

To examine expression, transformed protoplasts were incubated for 14 hr in the dark at room temperature with gentle shaking in the presence of 100 μCi ^{35}S protein labeling mixture (^{35}S-Met/Cys)(specific activity 1000 Ci/mmol to 1100 Ci/mmol)(Du Pont-New England Nuclear Research Products, Boston MA). Labeled proteins were chased for an additional 10 hr with an excess of unlabeled met and cys (final concentration of 15 mM and 7.5 mM, respectively). Protoplasts and incubation media were transferred to 1.5 ml microfuge tubes and separated by brief centrifugation (15-20 sec) at 800g. 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 fraction was prepared from the filtered incubation media as described in Bednarek et al. (1990) with the exception that 50 mg BSA was 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.

Plant Transformation

Tobacco plants (*Nicotiana tabacum* cv Wisconsin 38) were transformed with pGA643 Cuc Chit and Cuc Chit gene fusions as described in Wilkins et al., (1990). Axenic shoot cultures of transformed tobacco were maintained and propagated by node cuttings on solid MS medium without exogenous hormones.

Isolation and Radiolabeling of Transformed Tobacco Leaf Protoplasts

Protoplasts were prepared and isolated as described previously (Bednarek et al., 1990), with the exception that the cellulase/macerozyme mixture was prepared in 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). Protoplasts were purified by flotation in MS 0.1/1.0 medium supplemented with 0.6 M sucrose, washed once, and diluted to a final concentration of 400,000 protoplasts per milliliter in MS 0.1/1.0, 0.6 M betaine. Viable protoplasts were quantified (Bednarek et al., 1990) and labeled as described in Wilkins et al. (1990) with ^{35}S Met/Cys. Extracts of intracellular and extracellular proteins were prepared for immunoprecipitation as described above.

Vacuole Isolation

Protoplasts for vacuole isolation were prepared as described above. Vacuoles were released from the protoplasts by a combination of osmotic and thermal shock. Viable protoplasts (1 X 10^7) were chilled on ice for 30 min and then pelleted at 50g for 10 min at 4° C. Protoplast were gently lysed in lysis buffer (0.2

M sorbitol, 10% [w/v] Ficoll 400, 10 mM Hepes-KOH, pH 7.5, 10 μg/ml neutral red) and preheated to 45° C. Vacuoles were purified by flotation on a discontinuous Ficoll density gradient. The protoplast lysate was overlaid with two steps containing 5% [w/v] Ficoll 400 in 0.6 M betaine, 10 mM Hepes-KOH, pH 7.5, and 0.6 M betaine, 10 mM Hepes-KOH, pH 7.5; the gradients were centrifuged in a swinging bucket rotor at 5000g for 30 min at 4° C. Vacuoles were recovered from the 0%/5% (w/v) Ficoll 400 interface, quantitated using a hemocytometer, and gently lysed by osmotic shock. The vacuole suspension was diluted with 5 volumes of 10 mM Hepes-KOH, pH 7.5, and incubated at room temperature for 10 min. Membranes and unbroken vacuoles were cleared from the lysate by centrifugation at 100,000g for 30 min at 4° C. Soluble proteins were concentrated by ammonium sulfate (70% saturated at 20° C) and resuspended in 10 mM Hepes-KOH, pH 7.5, 0.5% (v/v) Triton X-100. For subcellular marker enzyme assays, extracts representing total protoplast proteins were prepared. Protoplasts were lysed in 10 mM Hepes-KOH, pH 7.5, 0.5% (v/v) Triton X-100 and cleared of insoluble material by centrifugation at 16,000g for 10 min at 4° C.

For immunoblot analysis, equal amounts of crude vacuole and protoplast lysate, relative to α-mannosidase activity, were precipitated with ice cold acetone (70% final concentration) for 1 hr at -20° C and collected by centrifugation at 16,000g at 4° C. Samples were resuspended in 30 μl SDS-PAGE sample buffer (50 mM Tris-HCl, pH 6.8, 2% [w/v] SDS, 1.0% [v/v] β-mercaptoethanol, 10% [v/v] glycerol, 0.01% [w/v] bromophenol blue), heated at 95° C for 5 min, and run on a 12.5% SDS-polyacrylamide gel. Gels were electroblotted onto Immobilon-P

Membrane (Millipore Corp., Bedford MA) (Towbin et al., 1979), and the membranes were blocked for 2 hr with TBS (20 mM Tris-HCl, pH 7.4, 150 mM NaCl) containing 3% (w/v) gelatin and 1% (w/v) BSA. The membranes were incubated for 1.5 hr with anti-Cuc Chit antiserum diluted 1:1250 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 1:5000 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).

Marker Enzyme Assays

NADH-cytochrome-c reductase was assayed by the method of Lord, (1983) with minor modifications. The assay (0.5 ml final volume) contained 20 mM potassium-phosphate buffer, pH 7.2, 0.5 mM NADH, 50 μM oxidized cytochrome c, 0.5% (v/v) Triton X-100. The NADH dependent reduction of cytochrome c was followed at 550 nm in a Beckman DU54 spectrophotometer (Beckman Instruments, Fullerton CA) at room temperature. The effects of 1 mM KCN and 1 μM antimycin on enzyme activity were investigated. Glucose-6-phosphate dehydrogenase was assayed as described by Simcox et al. (1977). α-Mannosidase was assayed as described by Boller and Kende (1979).

Immunoprecipitation and Analysis of Immunoprecipitated Proteins

Cuc Chit and Cuc Chit fusion proteins were purified by immunoprecipitation. To remove nonspecifically binding proteins, ^{35}S-labeled protoplast and media extracts were treated with 25 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-Cuc Chit antiserum was added to the cleared extracts and incubated at room temperature for 15 min. Immunocomplexes were collected on protein A-Sepharose CL-4B beads (Pharmacia, Piscataway NJ) for 15 min at room temperature and washed three times with TNET250. To further reduce nonspecific background, immunocomplexes were released from the protein A-Sepharose CL-4B beads by detergent solubilization with 1.0% SDS as described previously (Firestone and Winguth, 1990). The solubilized fraction was diluted in 1200 µl of TNET250 buffer with 0.5 mg BSA, and 0.6 µl anti-Cuc Chit antiserum and incubated at room temperature for 15 min with continuous mixing. Immunocomplexes were collected on protein A-Sepharose CL-4B beads washed once with TNET250 and once in nondetergent washing buffer (10 mM Tris-HCL pnnH 7.5, 5 mM EDTA). 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 (either 3-cm or 9-cm running gels) and visualized by fluorography as described previously (Mansfield et al., 1988). For treatment with endo H, immunopurified proteins were released from protein A-Sepharose CL-4B as described above. Samples were diluted fivefold in double distilled H_2O and

adjusted to 250 mM NaOAc pnnH 5.5, 1 mM phenylmethylsulfonylfluoride. The samples were incubated for 18 hr at 37°C in the presence of 5 milliunits endo H (Calbiochem Corp., La Jolla CA), precipitated with ice-cold acetone (final concentration 70%), and resuspended in 50 µl of SDS-PAGE sample buffer. As a positive control for endo H activity, affinity-purified proBL (Wilkins et al., 1990) was resuspended in 30 µl of SDS-PAGE sample buffer and treated as described above. Samples were analyzed on 12.5% SDS-polyacrylamide gels (9-cm running gel), and radiolabeled proteins were visualized by fluorography (Mansfield et al., 1988). Tunicamycin experiments were essentially performed as described in Mansfield et al. (1988) with the exception that the final concentration of tunicamycin was adjusted to 20 ug/ml.

Immunocytochemistry

All procedures were carried out at room temperature. Small pieces of leaf tissues from transgenic tobacco plants were fixed in the mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 10 mM sodium-phosphate buffer (pnnnH 7.2) with 0.1 M sucrose and vacuum infiltrated for 2 hr. After fixation the tissue was washed in the same buffer with 0.5 M sucrose three times, 10 min each. The tissue was postfixed in 1% OsO_4 in the same buffer with 0.05 M sucrose for 1 hr and then rinsed in distilled water three times, 5 min each. Following dehydration in an ethanol series, the tissue was embedded in London Resin White acrylic resin (Polysiences, Warrington PA) and polymerized at 60°C under vacuum overnight. Thin sections were prepared on an Ultracut E microtome (Reichert-Jung, Vienna

Austria) and mounted on formvar-coated nickel grids (Polysiences, Warrington PA). Immunocytochemistry was performed essentially as described by Herman and Melroy (1990). The primary antibody (rabbit anti-chitinase antiserum) was diluted 1 to 20, and control sections were incubated with nonimmune serum diluted similarly. Protein A-colloidal gold (EY Lab Inc., San Mateo CA) was diluted 1 to 50. Thin sections were examined on a JEOL 100CXII transmission microscope (Tokyo, Japan).

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CHAPTER 5

Vacuolar protein targeting in plants

INTRODUCTION

Protein transport to the lysosomes/vacuoles of plants, mammalian and yeast cells, is dependent on specific signals to divert lysosomal and vacuolar proteins from being secreted via the default pathway (for review see Chrispeels, 1991). The mechanisms involved in sorting and targeting of mammalian acid hydrolases to the lysosome have been extensively characterized (for review see Kornfeld and Mellman, 1989). In mammalian cells newly synthesized lysosomal enzymes are tagged by the phosphorylation of N-linked oligosaccharide mannose sidechains, and are sorted from secreted proteins in the TGN by receptors specific for mannose 6-phosphate (Man 6-P) residues (Kornfeld and Mellman, 1989). Ultimately, the information for sorting of these proteins is contained within the polypeptide sequence or structure of the protein which specifies phosphorylation of the glycan sidechain. In contrast to Man 6-P-dependent sorting of mammalian lysosomal hydrolases, targeting of plant and yeast proteins to the vacuole is dependent on the direct recognition of elements within the polypeptide sequence or structure (see Chrispeels, 1991 for review). In the last few years, a number of laboratories including ours have focused on the characterization of sorting signals from a number of different plant vacuolar proteins. A great benefit has been that each group examined a different protein, and what has emerged from this research, is that there is no **ONE** targeting signal for all plant vacuolar proteins. The sorting determinants for each protein or group of proteins may be tailored to the specific structure of the protein.

TONOPLAST PROTEINS

The existence of a wide variety of protein components in the tonoplast has been inferred from the many biochemical functions of the vacuole and demonstrated directly by analysis of tonoplast proteins (Dietz et al., 1988). The tonoplast must mediate a wide variety of functions including ATP-dependent acidification, the transport of ions and metabolites into and out of the vacuole as well as mechanisms for the docking and fusion of secretory transport vesicles. Whereas the structures of many of the soluble vacuolar proteins are known, the sequences of only a few tonoplast proteins, such as the 16 kDa polypeptide of the H^+-ATPase (Lai et al., 1991) and several isoforms of tonoplast intrinsic proteins (TIP) (Johnson et al., 1989; Höfte et al., in press) have been described. The deduced amino acid sequence of TIP shows a highly hydrophobic protein with six predicted membrane-spanning domains which has been proposed to function as a solute transporter (Johnson et al., 1990). To date, no information is available on the mechanisms by which the secretory pathway delivers membrane proteins to the tonoplast. Höfte et al. (1991) have demonstrated that the seed-specific TIP (αTIP) is targeted to the vacuoles of transgenic tobacco cells. This opens the way to identifying the sorting determinants necessary for tonoplast targeting of TIP and to comparing the mechanisms of tonoplast and soluble vacuolar sorting. The possibility remains that targeting to the tonoplast occurs by bulk-flow.

SORTING OF SOLUBLE VACUOLAR PROTEINS

Many plant vacuolar proteins are initially synthesized as precursors containing a propeptide domain which is proteolytically removed prior to or upon deposition of

the protein in the vacuole. Propeptides have been identified at either the amino- or carboxy-end of vacuolar proproteins which contain the information necessary to direct the protein to the vacuole. In addition the targeting information for other plant vacuolar proteins may be contained within the structure of the mature protein.

C-terminal Propeptide Sorting Signals

The Gramineae lectins from rice, wheat (WGA) and barley are soluble vacuolar proteins consisting of two identical subunits (reviewed in Raikhel and Lerner, 1991). The subunits dimerize within the lumen of the ER to form an active N-acetylglucosamine-binding proprotein containing two glycosylated carboxyl-terminal propeptides (CTPPs). Prior to or concomitant with deposition of the mature protein in the vacuole, the CTPPs are proteolytically removed. Wilkins et al. (1990) have demonstrated that barley lectin (BL) is properly processed and sorted in tobacco leaf cells, indicating that the endomembrane systems of monocots and dicots share a common mechanism for the posttranslational processing and sorting of vacuolar proteins (Wilkins et al., 1990). Deletion of the CTPP resulted in secretion of the BL via the default pathway demonstrating that the CTPP was necessary for vacuolar sorting of proBL (Bednarek et al., 1990). To examine whether the CTPP contained the essential topogenic information sufficient for vacuolar sorting of proBL, the CTPP was fused to the carboxy-terminus of an extracellular chitinase from cucumber (Métraux et al., 1989). Comparison of the intracellular and extracellular distribution of cucumber chitinase and the cucumber

chitinase-CTPP fusion protein in protoplasts prepared from transgenic tobacco plants indicated that the proBL CTPP redirected 70-75% of the normally secreted reporter protein to the vacuole (Bednarek and Raikhel, 1991). Further analysis to define the critical structural features or amino acids of the CTPP vacuolar targeting signal indicated that two independent amino acid stretches (FAEAI and LVAE) (see Table 1, highlighted) are each sufficient for sorting of proBL to the vacuole (J. Dombrowski, M. Schroeder, S.Y. Bednarek, and N.V. Raikhel, unpublished results). Identification of these short amino acid stretches as the critical elements of a targeting signal makes it unlikely that the predicted amphipathic α-helical structure of the CTPP (Bednarek et al., 1990) is required for sorting.

In contrast to the Gramineae lectins which are only found in the vacuole, chitinases have been localized in the extracellular spaces (Boller and Métraux, 1988) and in the vacuoles (Boller and Vögeli, 1984; Mauch and Staehelin, 1989) of plant cells. A comparison of the amino acid sequences of the extracellular acidic chitinases (class II) and the intracellular basic chitinases (class I) of tobacco reveals that the two isoforms share a large central domain, but that the vacuolar isoform contains two additional domains: 1) an amino-terminal domain, homologous to the small cysteine-rich protein from the latex of *Hevea brasiliensis*, hevein, and 2) a seven amino acid carboxyl-terminal (C-terminal) propeptide (Shinshi et al., 1990), which is processed prior to or upon deposition of the tobacco chitinase in the vacuole (Neuhaus et al., 1991). Deletion of the amino-terminal hevein-like domain of the vacuolar chitinase from *Nicotiana tabacum* did not affect the intracellular retention of the protein; however, removal of the C-

Table 1. Targeting motifs in representative C- and N-terminal propeptides

PROTEIN	SUFF./ NEC.	REF.	CARBOXYL-TERMINAL PROPEPTIDE	SEQ. REF.
Hordeum vulgare lectin	+/+	1	VF**AEAIA**ANST**LVAE**	2
Oryza sativa lectin	NA/NA	-	DGMAA**ILAN**NQSVSFEGIIES**VAELV**	3
Nicotiana tabacum chitinase	+/+	4	NG**LLVDTM**	4
N. tabacum ß-1,3-glucanase	NA/NA	-	VSGGVWDSSVETNATAS**LVSEM**	5
N. tabacum osmotin	NA/NA	-	YRVIFCPNGQAHPNFPIEMPGSDEVAK	6
Saponaria officinalis saporin 6	NA/NA	-	SSNEANSTVRHYGPLKPTLLIT	7
PROTEIN	SUFF./ NEC.	REF.	AMINO-TERMINAL PROPEPTIDE	SEQ. REF.
Ipomoea batatas sporamin A	+/NA	9	HSRFN**PIRLP**TTHEPA	10
H. vulgare aleurain	+/+	11	SSSSFAD**SNPIRPVTDRAAST**LE-/ /--DAAA	12
Solanum tuberosum 22 kDa protein	NA/NA	-	FTSENPIVLPTTCHDDN	13
S. tuberosum cathepsin D inhibitor	NA/NA	-	FTSQNLIDLPS	14
O. sativa oryzains	NA/NA	-	ASSGFDDSNPIRSVTDHAASA--/ /----DAPA	15

References for citations in Table 1

1) Bednarek and Raikhel, 1991; 2) see Raikhel and Lerner, 1991, for review; 3) Neuhaus et al., 1991; 4) Shinshi et al., 1990; 5) Shinshi et al., 1988; 6) Singh et al., 1989; 7) Benatti et al., in press; 8) Edens et al., 1982; 9) Matsuoka and Nakamura, 1991; 10) Hattori et al., 1985; 11) Holwerda et al., in press; 12) Rogers et al., 1985; 13) Yamagishi et al., 1991; 14) Strukeij et al., 1990; 15) Watanabe et al., 1991.

Abbreviations: SUFF., signal is sufficient for redirection of a reporter protein to the vacuole; Nec., signal is necessary for vacuolar protein sorting; Seq. Ref., sequence references; NA, no data available.

terminal propeptide resulted in secretion of the protein, as indicated by an increased level of chitinase activity in the intracellular wash fluid of transgenic *N. silvestris* leaves (Neuhaus et al., 1991). Furthermore, nine amino acids from the C-terminus of the basic chitinase (including the seven amino acid C-terminal propeptide) and a three amino acid linker region are sufficient for redirection of a cucumber chitinase fusion protein to the vacuole in transgenic tobacco plants (Neuhaus et al., 1991). Similar to the chitinases, the basic vacuolar isoform of ß-1,3-glucanases contains a C-terminal extension (Shinshi et al., 1988) not present in the acidic isoforms of ß-1,3-glucanases which are secreted into the extracellular leaf space in tobacco (Van den Bulcke et al., 1989; Payne et al., 1990). The vacuolar isoforms of β-1,3-glucanase from *N. tabacum* and *N. plumbaginifolia* are synthesized as a glycosylated precursors and are processed to their mature forms by removal of the glycosylated C-termini (Shinshi et al., 1988; Van den Bulcke et al., 1989). These results led Van den Bulcke et al. (1989) to postulate that the C-terminal extensions of the basic β-1,3-glucanases also contain the signals for vacuolar sorting of these proteins. The primary amino acid sequences of the vacuolar targeting signals from BL and the class I tobacco chitinase, and the C-terminal extensions from other vacuolar proteins are not conserved (Table 1). However, short amino acid stretches similar to the two small hydrophobic peptides (FAEAI and LVAE) contained in the proBL CTPP (see above) can be identified in the carboxyl-terminal extensions from WGA, rice lectin, the Class I chitinases from bean and tobacco, and the basic β-1,3-glucanase isoforms from tobacco (Table 1). These elements may form the critical core of the sorting signal which is

recognized by the vacuolar protein sorting machinery.

The C-terminal extensions from other plant proteins (Table 1) such as the type I ribosome inactivating protein Saporin 6 (Benatti et al., in press), and the vacuolar proteins osmotin (Singh et al., 1989) and thaumatin (Edens et al., 1982), appear to be removed, and may contain sorting information as well.

One of many responses of a plant cell to the invasion by pathogens (viruses, bacteria or fungi) is to induce the synthesis of a specific set of proteins known as the pathogenesis-related (PR) proteins (Van Loon, 1985). The most abundant PR protein, PR-1, is secreted into the leaf extracellular space in response to pathogen infection. Immunolocalization studies indicated that the PR-1 protein accumulated not only in the extracellular space but also in the vacuoles of specialized cells known as crystal idioblasts in leaves infected with tobacco mosaic virus and in the leaves of transgenic plants which are constitutively expressing only the PR-1 protein (Dixon et al., 1991). Although the mechanism(s) by which these specialized secreted proteins are delivered to the vacuoles of these cells are at this time only a matter of speculation (Dixon et al., 1991), these results suggest that mechanisms of protein sorting in plants may not only be mediated by specific topogenic signals but that it may also be regulated in a cell-specific manner.

N-terminal propeptide sorting signals

Many yeast and plant vacuolar proteins are synthesized as preproproteins with an amino-terminal propeptide domain which is exposed after cleavage of the signal sequence in the ER. The targeting information necessary and sufficient for

vacuolar localization of two yeast proteins, carboxypeptidase Y (CPY) and proteinase A, is contained within the amino-terminal propeptide of these proteins (Johnson et al., 1987; Valls et al., 1987; Klionsky et al., 1988).

Sporamin, the most abundant protein in the tuberous root of sweet potato, is initially synthesized as a preproprotein with an amino-terminal sequence containing a hydrophobic signal peptide and an additional 16 amino acid propeptide domain (Hattori et al., 1985) (see Table 1). In transgenic tobacco plants and suspension-cultured cells, prosporamin was correctly targeted to the vacuole (Matsuoka et al., 1990). Deletion of the amino-terminal propeptide resulted in secretion of sporamin by the transgenic tobacco cells (Matsuoka and Nakamura, 1991). Experiments to show that this propeptide is also sufficient for vacuolar sorting are in progress.

The barley thiol protease, aleurain is also processed by removal of an N-terminal propeptide prior to or concomitant with deposition of the protein in the vacuoles of barley aleurone cells (Holwerda et al., 1990) as well as in transgenic tobacco cells (Holwerda et al., in press). To identify the sorting determinant(s) necessary and sufficient for vacuolar targeting of proaleurain, Holwerda et al. (in press) made a series of chimeric proteins containing peptide segments from proaleurain fused to the N-terminus of another thiol protease, endoproteinase B (EP-B) which is normally secreted by barley aleurone cells (Koehler and Ho, 1990). A 12 amino acid peptide (SSSSFADSNPIR) (Table 1) contained within the N-terminal aleurain propeptide redirected 52% of the EP-B to the vacuole in transgenic tobacco (Holwerda et al., in press). Comparison of the sporamin N-

terminal propeptide and this sequence reveals a conserved sequence (NPIR) (Table 1, highlighted). Substitution of either the I or N with glycine resulted in secretion of prosporamin (K. Matsuoka and K. Nakamura, personal communication), indicating that these amino acids are critical residues of the sporamin vacuolar targeting signal. Other secretory proteins have been identified which possess N-terminal propeptides with sequences similar to the NPIR element found in proaleurain and prosporamin (Table 1) which may also be necessary for sorting of these proteins. Deletion of the amino acids SNPIR from the proaleurain N-terminal propeptide lowered the efficiency of vacuolar sorting of aleurain by 65% (Holwerda et al., in press), suggesting that these residues are also critical for proaleurain sorting. In addition to the SNPIR element, the flanking amino acid segments SSSSFAD and VTDRAAST (see Table 1) were necessary for the efficient sorting of aleurain (Holwerda et al., in press). These results led Holwerda et al. (in press) to suggest that proaleurain vacuolar sorting signal may be composed of multiple domains that collectively mediate the efficient vacuolar targeting of this protein. Alternatively, these flanking sequences may present the targeting signal in a favorable three dimensional structure for interaction with the vacuolar protein targeting mechanism; such that disruption of this structure would lower the efficiency of sorting. The availability of the sorting information may be altered in fusion proteins containing targeting determinants from other proteins and may explain the less than 100% efficient redirection of reporter proteins to the vacuole by the BL CTPP (Bednarek and Raikhel, 1991) and the aleurain targeting determinant (SSSSFADSNPIR) (Holwerda et al., in press).

Internal targeting signals

Many soluble plant vacuolar proteins are synthesized without a cleavable propeptide, indicating that the vacuolar targeting information for these proteins must be contained within the mature protein. This information could consist of a linear sequence of amino acids from two or more regions of the polypeptide chain that are situated closely together in the three-dimensional structure of the protein. Analysis of the mechanisms of sorting of PHA (Tague et al., 1990), 11S legumin (Saalbach et al., 1991) and patatin (Sonnewald et al., 1991), has shown that all these proteins possess long internal polypeptide regions with vacuolar targeting information. A segment of the PHA polypeptide (amino acids 83 to 146) was sufficient and necessary to redirect 50% of the secreted protein invertase to the vacuole in *Arabidopsis* protoplasts (A. von Schaewen and M. Chrispeels, personal communication). Examination of the crystal structure of other legumin lectins (Cunningham et al., 1975) which are homologous to PHA, suggests that the identified PHA targeting determinant contains a loop (amino acid 93-115) present at the surface of the molecule. This loop is likely to present the sorting information of PHA in a accessible structural context necessary for interaction with the vacuolar sorting machinery.

Mechanisms of plant vacuolar protein sorting

The above reviewed data demonstrate that the sorting information for vacuolar proteins can be contained within C-, N-terminal propeptides or within internal regions of the mature protein. In addition the structure of the protein affects the

availability of the sorting signals to interact with the secretory machinery and is an important factor for efficient vacuolar protein targeting. Interestingly, double immunolocalization of ultrathin sections obtained from crossed plants expressing BL (C-terminal sorting signal) and sporamin (N-terminal sorting signal) indicated that both proteins are targeted to the same vacuoles in leaves and roots of transgenic tobacco plants (M. Schroeder, O. Borkhsenious, K. Matsuoka, K. Nakamura, and N.V. Raikhel, unpublished results). Although the primary amino acid sequence of C- and N-terminal sorting determinants are not conserved (Table 1) the results of the double immunolablelling experiments suggest that both sorting signals are recognized by the machinery present in the same cell. The variability of these vacuolar sorting signals suggests that there may be multiple independent mechanisms for vacuolar protein sorting in plants. It is possible however, that some physicochemical properties of these propeptides are recognized by a common sorting mechanism.

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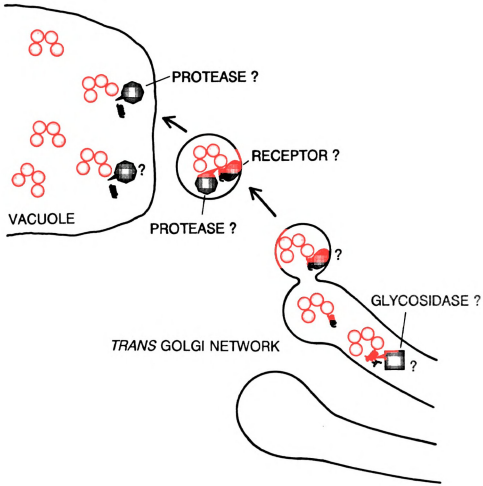
CHAPTER 6

Future Research Prospectives

Conventional models of protein sorting require specific sorting signals and a membrane bound receptor or a series of soluble and membrane bound receptors with specificity for the targeting signal (see Figure 1), similar to those specific for the Man-6-P tagged lysosomal hydrolases (Kornfeld and Mellman, 1989) or those involved in the targeting of newly synthesized polypeptides to the ER (Rapoport, 1990), respectively. In contrast to a receptor mediated processes, sorting of regulated secretory proteins in mammalian cells is postulated to occur by the selective aggregation of these specific proteins in the *trans*-golgi network (TGN) as a result of the chemical environment (a low pH and high calcium concentration) within this compartment (Burgess and Kelly, 1987; Tooze et al., 1989; Chanat and Huttner, 1991).

Vacuolar proteins are diverted from the flow of secretory proteins to the cell surface and targeted to the vacuole by specific signals contained in the polypeptide sequence or in its three dimensional structure. The information necessary and sufficient to target barley lectin to the plant cell vacuoles is contained in a 15 amino acid carboxyl-terminal propeptide (CTPP) (Bednarek et al., 1990; Bednarek and Raikhel, 1991). Preliminery experiments to further characterize the amino acid residues of the CTPP critical for vacuolar protein sorting have identified two independent hydrophobic amino acid stretches interspersed with charged residues (FAEAI and LVAE) (see Table 5.1, highlighted). Each stretch was found to be sufficient for sorting of proBL to the vacuole (J. Dombrowski, M. Schroeder, S.Y. Bednarek, and N.V. Raikhel, unpublished results).

FUTURE RESEARCH GOALS



Similarly, the C-terminal sorting signal of the tobacco basic chitinase and the C-terminal propeptides from other plant vacuolar proteins also contain short stretches of hydrophobic amino acids interspersed with charged residues (see Chapter 5), suggesting that these proteins may all be targeted to the vacuole by a common mechanism in both monocots and dicots. A putative receptor for the C-terminal vacuolar sorting determinants signals would presumably recognize some common physicochemical characteristic such as the short hydrophobic amino acid peptides since the primary amino acid sequences of these signals are not conserved. Alternatively, these short hydrophobic patches could be involved in the selective aggregation of vacuolar proteins carrying these signals. Studies with the sodium ionophore monensin on the processing of barley lectin suggest that the sorting of the proprotein occurs in the *trans*-Golgi or TGN (Wilkins et al., 1990). In addition to question regarding the mechanism by which vacuolar proteins are segregated from secreted proteins (ie receptor vs aggregation mediated sorting), many other unknowns remain such as: how sorting of these proteins is coupled to, or triggers the formation of transport vesicles destined for the vacuole and what factors distinguish these vesicles from those which carry secreted proteins? Although some of the steps and components involved in the budding and attachment of secretory transport vesicles have been elucidated (for review see Rothman and Orci, 1992) the mechanisms by which these vesicles are targeted to and fuse with another compartment are unknown.

Many other questions involving the processing of the barley lectin proprotein remain unanswered. Is the glycosylated CTPP removed from the

proprotein to yield the mature vacuolar protein in a single step or by a series of processing steps? Pulse-chase analysis of the rate of CTPP cleavage in *wt* and *gly* barley lectin proprotein indicated that the rate of processing of the *gly* was faster than the *wt* (Wilkins et al., 1990) indicating that the glycan reduced the availability of the propeptide to interact with processing enzymes. Wilkins et al. (1990) hypothesized that removal of the CTPP initially requires cleavage of the glycan side chain by a putative glycosidase from the propeptide. An intermediate processed form of probarley lectin containing a deglycosylated propeptide however has not been detected *in vivo* suggesting that removal of the propeptide occurs very rapidly upon deglycosylation of the CTPP. Alternatively, the glycan side chain could reduce or hindered the accessibility of the propeptide to the sorting machinery. The higher rate of processing of the *gly* proprotein could result from a faster rate of sorting to a compartment containing the processing enzymes. Questions therefore of how many steps are involved in processing of the CTPP and in which compartment they occur are still open.

Many of the tools to study these questions are available. The proper sorting of plant vacuolar proteins in heterologous plant species allows for the development of genetic selection schemes in *Arabidopsis* to isolate genes involved in the sorting and processing of secretory proteins. The production of antibodies which recognize components of the sorting machinery will be used to identify their subcellular localization. Furthermore, cDNAs encoding the light and heavy chains of these antibodies can be expressed in plants (Hiatt et al., 1989) and used to examine their effect on protein sorting *in vivo*. Conserved proteins involved in

secretory protein transport in yeast and mammalian cells may also provide probes with which to identify their plant homologues and to clone them. Identification of the vacuolar sorting determinant of barley lectin is only the first step to understanding the mechanism of vacuolar protein sorting in plants.

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