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A Carboxyl-Terminal Propeptide Is Necessary for Proper Sorting of Barley Lectin to Vacuoles of Tobacco

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Barley lectin is synthesized as a preproprotein with a glycosylated carboxyl-terminal propeptide (CTPP) that is removed before 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, T.A., Bednarek, S.Y., and Raikhel, N.V. (1990). 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, T.A., Bednarek, S.Y., and Raikhel, N.V. (1990). Plant Cell 2, 301–313]. 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 the mutant barley lectin was 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 an N-terminal hydrophobic signal sequence that mediates a transmembrane translocation from the cytosol to the lumen of the ER (Blobel and Dobberstein, 1975). After proteolytic cleavage of the signal sequence, some secretory proteins undergo further post-translational processing in the ER and Golgi network. Proteins traversing the secretory pathway are believed to be sorted to their respective compartments by selective retention or by 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 (Blobel, 1980). 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 some mammalian lysosomal enzymes (Gabel et al., 1983; Williams and Fukuda, 1990). 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; Sonnewald et al., 1990; Wilkins 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. It has been demonstrated that the sorting determinant of the yeast vacuolar carboxypeptidase Y (CPY) is contained within the amino terminus of the preproprotein (Johnson et al., 1987; Valls et al., 1987). A detailed mutational analysis of the amino-terminal propeptide determined that the tetrapeptide Gln-Arg-Pro-Leu functions as a vacuolar sorting signal. Interestingly, the context in which the Gln-Arg-Pro-Leu 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). Another sorting determinant was identified in the amino-

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terminal propeptide of the yeast vacuolar enzyme, proteinase A (Klionsky et al., 1988), which is sufficient to redirect the normally secreted enzyme invertase to the yeast vacuole. However, currently, no consensus 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.

The plant vacuolar protein phytohemagglutinin-L (PHA), a lectin of common bean, is correctly processed and sorted to the yeast vacuole (Tague and Chrispeels, 1987). In addition, a PHA fusion protein containing the secreted form of yeast invertase is redirected to the yeast vacuolar compartment (Tague and Chrispeels, 1989). Deletion analysis of PHA 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 (Leu-Gln-Arg-Asp) that 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 successfully targeted to vacuoles in *Arabidopsis thaliana* protoplasts (Chrispeels, 1991). Therefore, this sorting determinant contains sufficient 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 that are initially synthesized as glycosylated 23-kD polypeptides that dimerize within the lumen of the ER to form an active *N*-acetylglucosamine (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 plant 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 CTPP N-linked glycan revealed that although the glycan is not essential for processing and vacuolar sorting of the barley lectin in tobacco, the presence of the glycan does modulate the rate of processing of the propeptide (Wilkins et al., 1990).

Although the primary sequences of the CTPPs 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 CTPP by examining the assembly and sorting of a barley lectin mutant lacking the CTPP. We have established transient expression and stably transformed suspension-cultured cells systems, in addition to transgenic plants, to facilitate the analysis of the vacuolar sorting of barley lectin. Using these 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 CTPP of Barley 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 cotranslationally glycosylated. The proprotein consists of four highly homologous domains of 43 amino acids and a 15-amino acid CTPP that contains an N-linked high-mannose glycan. Before 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 of the cDNA clone pBlc3 (Lerner and Raikhel, 1989) was deleted (see Methods) 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 the CTPP. 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 were designated by the code *ctpp*$^-$, as shown in 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

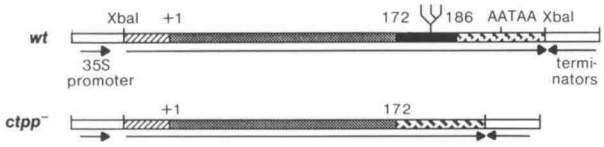

Figure 1. Organization of the Wild-Type (*wt*) and Carboxyl-Terminal Mutant (*ctpp*$^-$) Barley Lectin cDNAs.

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 through 186). The 15-amino acid CTPP of barley lectin proprotein (amino acids 172 through 186) contains an N-linked high-mannose-type glycan, as depicted by the branched structure.

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 by way of polyethylene glycol treatment (Negrutiu et al., 1987), and the protoplasts were pulse labeled for 12 hr 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 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 pulse-labeled NT protoplasts expressing the *wt* construct (Figure 2, lane 2). Radiolabeled barley lectin was not detected in the incubation media recovered from NT protoplasts expressing *wt* barley lectin (Figure 2, lane 5). Similarly, barley lectin is not detected in the incubation media of ^{35}S-met/cys-labeled leaf protoplasts from *wt* 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 (Figure 2, lane 3). Deletion of the CTPP resulted in the appearance of radiolabeled barley lectin in the incubation media of the protoplasts transiently expressing only the *ctpp*$^-$ construct (Figure 2, lane 6). These results support the observation (Peumans et al., 1982) that the subunits of barley lectin do not require the CTPP to dimerize correctly 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 NT Cells and Plants

To investigate further the role of the CTPP in the sorting of barley lectin to vacuoles, tobacco plants and NT 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, NT cells offer many advantages for the analysis of protein sorting. NT cells can be transformed readily at a high frequency by cocultivation with *Agrobacterium tumefaciens* and the resulting kanamycin-resistant transformants analyzed within 7 weeks to 8 weeks after selection. The individual nature of NT cells and their immediate contact with the surrounding media allow for the direct analysis of protein secretion from transformed cells.

NT cells were transformed by cocultivation with *A. 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*.

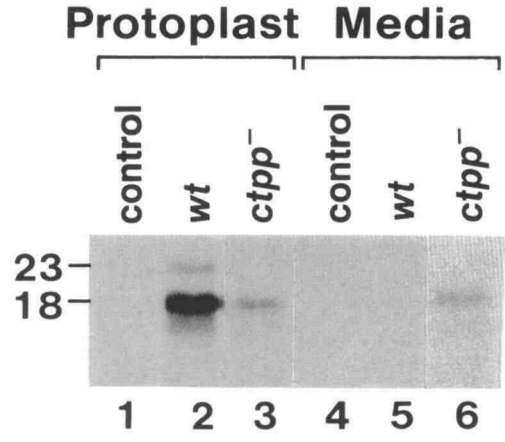

Figure 2. Protein Gel Blot Analysis of Transiently Expressed *wt* and *ctpp*$^-$ Barley Lectin cDNA Constructs.

Protoplasts from NT cells were transiently transformed with *wt* and *ctpp*$^-$ pGA643 constructs by way of 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 separated 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.

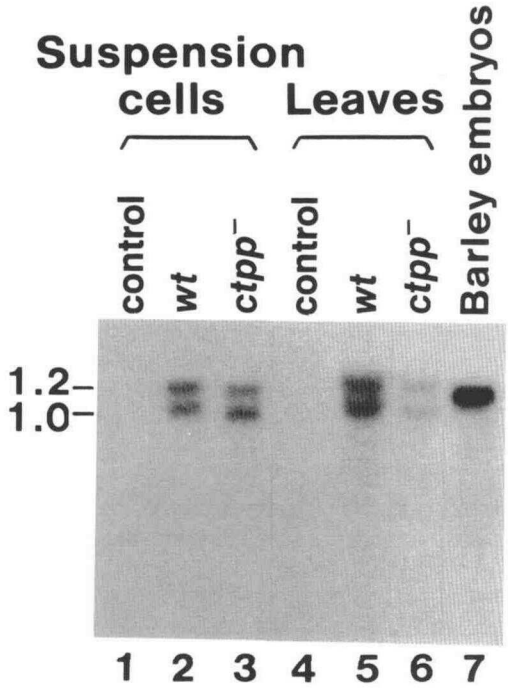

Figure 3. Accumulation of Steady-State mRNA Levels of Barley Lectin in NT Cells and in Transgenic Tobacco.

RNA gel blot analysis of total RNA from NT cells or tobacco leaves. Total RNA (20 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), and transgenic tobacco plants containing *wt* (lane 5) or *ctpp*⁻ (lane 6) barley lectin cDNA constructs were separated by electrophoresis on a 2% agarose/6% formaldehyde gel and analyzed as described in Methods. Total RNA (10 μg) isolated from developing barley embryos (lane 7) serves as a positive control. The sizes of the *wt* barley lectin mRNA species (in kilobases) are shown on the left.

The steady-state levels of *wt* and *ctpp*⁻ barley lectin mRNA in transgenic NT cells and tobacco plants were compared using 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 the *wt* and *ctpp*⁻ transformants examined in this paper. Two mRNA species of 1.2 kb and 1.0 kb were observed in total RNA from *wt* transformants (Figure 3, lanes 2 and 5). Two slightly smaller mRNA species of 1.15 kb and 1.05 kb were detected in total RNA from *ctpp*⁻ transformants (Figure 3, lanes 3 and 6). 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 (Figure 3, lanes 1 and 4). The relative levels of *ctpp*⁻ barley lectin mRNA were twofold to threefold lower than corresponding *wt* mRNA in transformants, as determined by scanning densitometry (Figure 3, lanes 5 and 6). 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).

The CTPP Mutant of Vacuolar Barley Lectin Is Secreted in NT 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 that lack or have altered targeting signals cannot be recognized by the vacuolar protein sorting machinery and will be secreted by way of 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 NT cells, NT-*wt* and NT-*ctpp*⁻ cells were pulse labeled for 6 hr in the presence of ^{35}S-met/cys and chased for an additional 10 hr 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

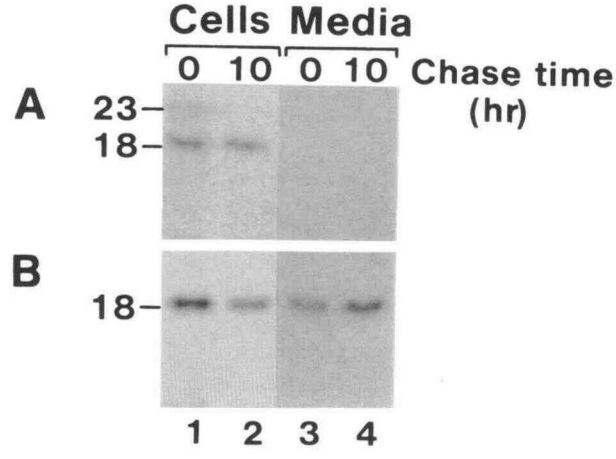

Figure 4. Secretion of Barley Lectin from NT Cells Transformed with *wt* and *ctpp*⁻ Constructs.

(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 from cells and incubation media. Affinity-purified proteins were analyzed by SDS-PAGE on 12.5% polyacrylamide gels and fluorography. The molecular masses (in kilodaltons) of the *wt* precursor (23 kD) and mature subunit (18 kD) are shown on the left.

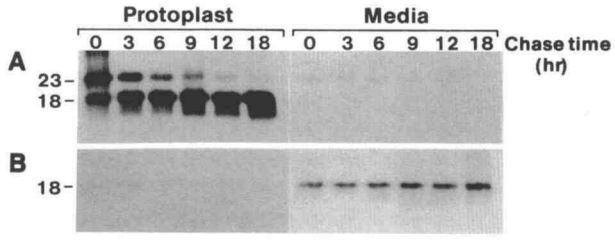

Figure 5. Pulse-Chase Labeling Experiments of Tobacco Protoplasts Isolated from Transformed Tobacco Plants Expressing *wt* or *ctpp*$^-$ Barley Lectin.

(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. Protein 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 masses (in kilodaltons) of the *wt* barley lectin precursor (23 kD) and mature subunit (18 kD) are displayed on the left.

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 (Figure 4A, lane 1). During the 10-hr 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 (Figure 4A, lane 2). Neither the labeled precursor nor the mature polypeptide of barley lectin was present in the NT-*wt* incubation media during the 10-hr chase period (Figure 4A, lanes 3 and 4). However, the 18-kD mature polypeptide of barley lectin was detected in both the NT-*ctpp*$^-$ cells (Figure 4B, lane 1) and incubation media (Figure 4B, lane 3). During the 10-hr chase period, there was a decrease in the level of intracellular 18-kD polypeptide (Figure 4B, lane 2) and a corresponding increase in the amount of 18-kD barley lectin subunit in the media (Figure 4B, lane 4). Radiolabeled 18-kD subunit was still present in the NT-*ctpp*$^-$ cells after a 10-hr chase.

To extend the analysis of *ctpp*$^-$ barley lectin secretion, W38-*wt* and W38-*ctpp*$^-$ leaf protoplasts were pulse-labeled for 10 hr in the presence of ^{35}S-met/cys. Labeled proteins were chased with unlabeled met/cys for an additional 18 hr. 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 (Figure 5A, 0 hr), both the 23-kD barley lectin proprotein and the 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 the 18-kD mature subunit correspondingly increased. However, some 23-kD precursor was still visible after 18 hr of chase in the *wt* protoplasts (Figure 5A, 18 hr), indicating the continued low-level incorporation of labeled amino acids into newly synthesized polypeptides. After 10 hr of pulse labeling, mature 18-kD polypeptides derived from the CTPP barley lectin mutants 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 hr of chase, some 18-kD polypeptides 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 hr of exposure and not in vacuoles from *ctpp*$^-$ transformants (data not shown). However, after a 14-day exposure of the same gel, another band corresponding to the 23-kD precursor was visible 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 suggested that the *wt* vacuole preparation was contaminated with ER and Golgi organelles. Therefore, it was difficult to assess whether the presence of radiolabeled barley lectin in the *ctpp*$^-$ vacuoles was the result of some remaining vacuolar targeting of barley lectin lacking the CTPP or whether it resulted from contamination of the vacuole preparation by ER and Golgi compartments.

In this experiment, a low level of the 23-kD proprotein was discernible in the incubation media of W38-*wt* protoplasts. The absence of any detectable 18-kD subunit in the media indicated that the presence of the glycosylated proprotein was 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 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) that 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). The CTPPs shown in Figure 6 are predicted by computer analysis of protein secondary structure to form amphipathic α-helices (Wilkins and Raikhel, 1989). In contrast to the 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 CTPP in vacuolar sorting of barley lectin, we have expressed a CTPP deletion mutant of barley lectin in tobacco protoplasts, transgenic NT cells, and transgenic plants. Using these three different systems, we have demonstrated that 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 hr 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 demonstrated 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 suggest that the CTPP 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 before or upon arrival of the proprotein to vacuoles. Similar to the Gramineae lectins, the vacuolar isoforms of β-1,3-glucanases of tobacco and *Nicotiana plumbaginifolia* are initially synthesized as glycosylated precursors and processed into their mature forms by the removal of a glycosylated CTPP (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 because of acidic amino acids. Features such as the acidic

BARLEY lectin	V F A E A I A A N* S T L V A E
WGA-A	V F A E A I T A N* S T L L Q E
WGA-B	V F A E A I A T N* S T L L A E
WGA-D	V F A G A I T A N* S T L L A E
RICE lectin	D G M A A I L A N N* G S V S F E G I I E S V A E L V
N. tabacum β-1,3-glucanase	V S G G V W D S S V E T N* A T A S L V S E M
N. plumbaginifolia β-1,3-glucanase	F S D R Y W D I S A E N N* A T A A S L I S E M

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

With the exception of the WGA isolectins and barley lectin, the primary amino acid sequences of the CTPPs of the Gramineae lectins (Raikhel and Wilkins, 1987; Lerner and Raikhel, 1989; Smith and Raikhel, 1989; Wilkins and Raikhel, 1989) and the intracellular isoforms of tobacco (Shinshi et al., 1988) and *N. plumbaginifolia* (De Loose et al., 1988) β-1,3-glucanase are not conserved. The stars (★) above the residues denote the utilized N-linked glycosylation sites. Acidic residues (E, glutamic acid; D, aspartic acid) are highlighted in boldface type and underlined.

nature of these glycopeptides and/or secondary structure may be important in the molecular mechanisms of vacuolar sorting for these proteins.

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 isoforms are initially synthesized with or without a CTPP. Recently, another β-1,3-glucanase cDNA clone from tobacco (Neale et al., 1990) was isolated. This clone is homologous to 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, 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 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 (Chrispeels, 1983; Tartakoff, 1983). In the presence of monensin, cleavage of the CTPP and transport of the barley lectin precursor from the Golgi are 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 shown in Figure 7. The final step in the maturation of barley lectin has not been precisely characterized. It remains unknown whether the CTPP 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). However, 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 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 either very fast or that processing of the glycosylated CTPP actually occurs in a

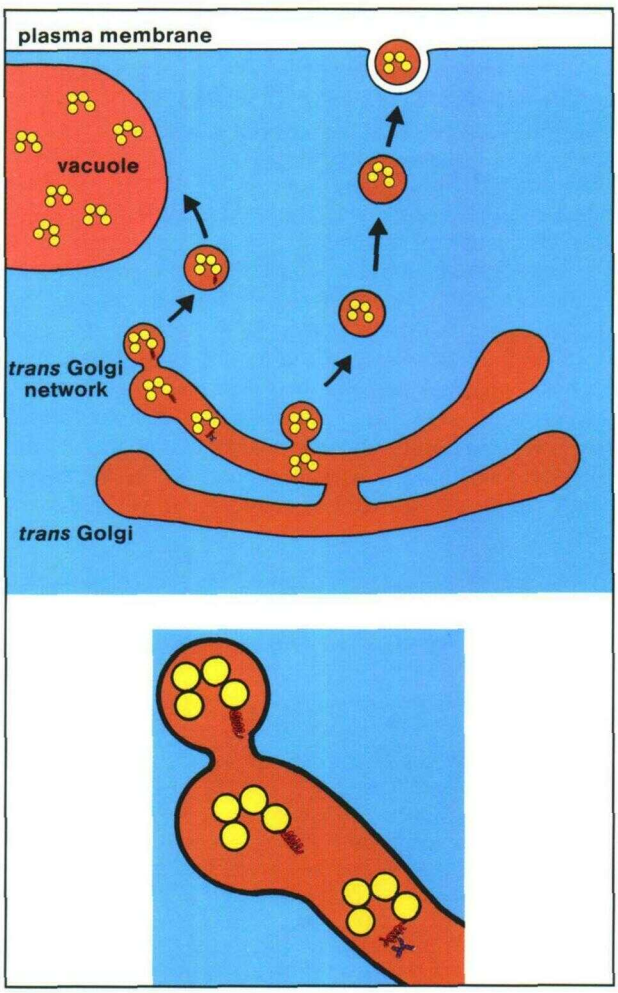

Figure 7. Model for Barley Lectin Sorting in the *trans*-Golgi Network.

The schematic representation of one subunit of a barley lectin dimer was adapted from the crystal structure of WGA (Wright, 1987). Each of the four highly homologous domains of barley lectin is represented by a small circle. The glycosylated CTPP is depicted as a red 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). (Area has been enlarged to show detail.) The CTPP of barley lectin is necessary for sorting of this protein to vacuoles. The barley lectin mutant lacking this sorting signal is secreted.

single step. The single-step processing 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 by way of secretory vesicles. For example, the *ctpp*$^-$ barley lectin mutant lacking the sorting signal was secreted (Figure 7). Similarly, overproduction of a vacuolar protein may saturate the sorting pathway, thereby resulting in the secretion of the protein by way of 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 that deglycosylates the proprotein or the sorting apparatus that recognizes the CTPP and targets barley lectin to plant vacuoles.

METHODS

Preparation of Barley Lectin Propeptide Deletion Mutants

Nucleotides 607 to 651 of the barley lectin cDNA (Lerner and Raikhel, 1989), which encode the CTPP 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 Messing, 1987). The synthetic mutagenic oligonucleotide 5′-CGGCGGCTGCGACGGT/TGATGATCTTGCTAATGGCAG-3′ (nucleotides 591 to 606/nucleotides 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 CTPP deletion mutants of barley lectin were identified and selected as described by Wilkins et al. (1990), subcloned 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.

NT Cell and Shoot Tissue Culture

Nicotiana tabacum suspension-cultured (NT) cells were maintained in liquid Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 0.2 mg/L 2,4-D (MS 0.2 mg/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 to fresh media. Axenic shoot cultures of *N. tabacum* (cv Wisconsin 38) were maintained and propagated by node cuttings on solid MS medium.

Transient Gene Expression System in Tobacco Suspension Protoplasts

Protoplasts were prepared from 3-day NT cell cultures. NT cells were collected by centrifugation at 50*g* for 5 min at room temperature. The cell pellet was resuspended and digested in MS 0.2 mg/L 2,4-D with 1.0% cellulase Onozuka R10, 0.5% macerozyme R10 (Yakult Honsha Co., Ltd., Shingikan-CHO, Nishinomiya, Japan), 0.1% BSA, and 0.4 M sucrose at 28°C for 4 hr with gentle shaking on a gyratory shaker at 75 rpm. Protoplasts were filtered through a 90-μm steel mesh screen and purified by centrifugation in Babcock bottles (Baxter Scientific Products, McGaw Park, IL) at 350*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 $CaCl_2 \cdot 2H_2O$, 5 mM KCl, 5 mM 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*g* for 10 min and resuspended to a final concentration of 1.7×10^6 viable protoplasts/mL with MaMg solution (0.4 M mannitol, 15 mM $MgCl_2$, 3 mM Mes-KOH, pH 5.6) (Negrutiu et al., 1987). Before adding plasmid DNA, 5×10^5 protoplasts were aliquoted to 15-mL polypropylene tubes (300 μL of a 1.7×10^6 protoplasts/mL 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 constructs (no plasmid in negative control) and 50 μg of sheared salmon sperm DNA was added to the protoplast suspension. The protoplast/plasmid DNA mixture was brought to a final concentration of 28% PEG-4000 with a solution containing 40% PEG-4000, 0.4 M mannitol, 100 mM $Ca(NO_3)_2 \cdot 4H_2O$, 10 mM 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*g* for 10 min at room temperature and the protoplast pellet was resuspended in 2.5 mL of MS 0.2 mg/L 2,4-D and 0.4 M mannitol to a final density of 2.0×10^5 protoplasts/mL and transferred to 80 × 15-mm Petri plates.

To examine expression of the barley lectin constructs, the transiently transformed NT protoplasts were incubated for 12 hr in the presence of 200 μCi of Expre^{35}S^{35}S ^{35}S protein labeling mixture (Du Pont-New England Nuclear Research Products), *E. coli* hydrolysate containing a mixture of 77% L-^{35}S-methionine and 18% L-^{35}S-cysteine in 50 mM tricine, 10 mM β-mercaptoethanol buffer (specific activity 1000 Ci/mmol to 1100 Ci/mmol) (^{35}S-met/cys). After labeling, the protoplasts were separated from the culture medium by centrifugation at 50*g* for 10 min at room temperature. The protoplast pellet was resuspended in 200 μL of 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*g* for 5 min at 4°C, frozen in liquid nitrogen, and stored at −70°C. The culture medium (2.5 mL) was filtered to remove any remaining protoplasts, as described in Wilkins et al. (1990). Proteins in the culture medium were precipitated with ammonium sulfate at 70% saturation at 4°C for 2 hr and then collected by centrifugation at 12,000*g* for 10 min at 4°C. The culture medium protein pellet was resuspended in 200 μL of extraction buffer and stored at −70°C.

All protein samples were thawed at room temperature and passed four times over immobilized GlcNAc (Pierce) microaffinity columns (Mansfield et al., 1988). After extensive washing of the column with TA buffer (50 mM Tris acetate, pH 5.0, 100 mM NaCl), barley lectin was eluted with 150 μL of 100 mM GlcNAc 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).

NT Cell and Plant Transformation

The binary vector pGA643 constructs containing *wt* or *ctpp*$^-$ were mobilized to *Agrobacterium tumefaciens* LBA4404 as described in Wilkins et al. (1990). NT suspension cells were cocultivated with agrobacteria harboring *wt* and *ctpp*$^-$ pGA643 constructs according to An (1985) and plated on MS 0.2 mg/L 2,4-D agar supplemented with 500 mg/L carbenicillin and 150 mg/L kanamycin. After 3 weeks to 4 weeks, calli 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 NT cells and plants as described (Nagy et al., 1988). Twenty micrograms of total RNA from each sample was fractionated on 2% agarose gels containing 6% formaldehyde and blotted onto 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 NT Cells

For pulse-chase labeling experiments, 0.5 mL of NT cells (per well) from 4-day-old cultures were incubated in 24-well Falcon tissue culture plates in the presence of 85 μCi of ^{35}S-met/cys (see above). Two wells or a total of 1 mL of the 4-day NT cells were labeled per timepoint. The cells were incubated at room temperature with gentle shaking on a gyrotary shaker at 75 rpm in the dark for 6 hr. After 6 hr, labeled proteins were chased by adding unlabeled methionine and cysteine to a concentration of 5 mM and 2.5 mM per well, respectively. At the appropriate timepoints, labeled NT cells were pooled in 1.5-mL microcentrifuge tubes and the cells were separated from the media by centrifugation at 2000*g* for 1 min at 4°C.

The culture media were transferred to another tube and centrifuged at 16,000*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 of MS 0.2 mg/L 2,4-D and pelleted by centrifugation at 2000*g* for 1 min at 4°C. Cells were homogenized in 300 μL of 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 nitrogen and the ice slurry was homogenized using a motor-driven microcentrifuge pestle (Kontes Co., Vineland, NJ). The homogenate was centrifuged at 16,000*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

Protoplasts for labeling were prepared from fully expanded leaves of 4-week-old to 6-week-old axenic shoot cultures of W38-*wt* and W38-*ctpp*$^-$ barley lectin transformants. Leaf protoplasts were prepared as described in Wilkins et al. (1990) with the exception that the enzyme mixture was dissolved in MS medium supplemented with 1.0 mg/L benzyladenine (BA), 0.1 mg/L naphthaleneacetic acid (NAA), and 0.6 M mannitol. To remove broken protoplasts and undigested cells, the protoplasts were pelleted at 50*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*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. Leaf protoplasts were labeled as described in Wilkins et al. (1990).

Vacuole Isolation from Labeled Tobacco Leaf Protoplasts

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

Vacuoles were isolated as described (Wilkins et al., 1990) with minor modifications 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*g* at 4°C. Soluble proteins were concentrated by precipitation with ammonium sulfate at 70% saturation at 4°C for at least 2 hr. Precipitated proteins were collected by centrifugation for 10 min at 16,000*g* at 4°C. The protein pellet was resuspended in 300 μL of 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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