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Role of Propeptide Glycan in Post-Translational Processing and Transport of Barley Lectin to Vacuoles in Transgenic Tobacco

Thea A. Wilkins,[1] Sebastian Y. Bednarek, and Natasha V. Raikhel[2]

Department of Energy Plant Research Laboratory, Michigan State University, East Lansing, Michigan 48824-1312

Mature barley lectin is a dimeric protein composed of two identical 18-kilodalton polypeptides. The subunits of barley lectin are initially synthesized as glycosylated proproteins, which are post-translationally processed to the mature protein preceding or concomitant 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* and *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.

INTRODUCTION

Many proteins entering the endomembrane system of the secretory pathway are modified in the lumen of the rough endoplasmic reticulum (ER) by the covalent attachment of high-mannose oligosaccharide sidechains (glycans) to selective asparagine (N) residues. The N-linked high-mannose glycans subsequently may be 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, 1986).

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 (Akazawa and Hara-Nishimura, 1985; Bollini et al., 1985; Voelker et al., 1989). 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 vacuolar protein concanavalin A (Con 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 (pro-ConA) (Herman et al., 1985). The mature ConA polypeptide is generated by the excision of an internal glycopeptide from pro-ConA 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 pro-ConA 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

[1] Current address: Department of Agronomy and Range Science, University of California, Davis CA 95616.

[2] To whom correspondence should be addressed.

rice are 36-kD dimers assembled from two identical 18-kD subunits (Rice and Etzler, 1974; Peumans et al., 1982a, 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 (T.A. Wilkins and N.V. 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; T.A. Wilkins and N.V. Raikhel, unpublished results). The COOH-terminal N-glycopeptide of the proprotein is post-translationally removed before or concomitant with deposition of the mature protein in vacuoles. The transient glycosylation of the Gramineae lectin proproteins provides a unique opportunity to investigate the molecular mechanisms that 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 rough ER/Golgi complex were distinctive for the wild-type or mutant barley lectin proproteins.

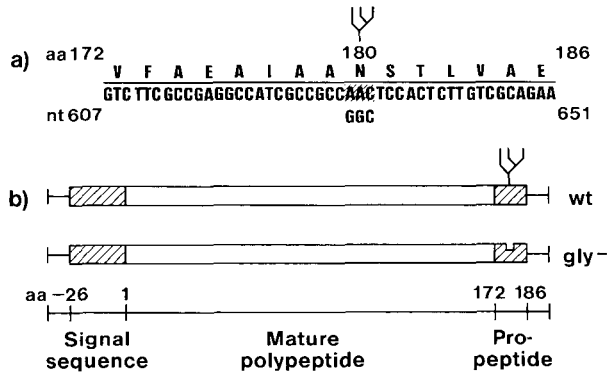


Figure 1. Alteration of the N-Linked Glycosylation Site of Barley Lectin by Site-Directed Mutagenesis and Organization of the Wild-Type (*wt*) and Mutant (*gly*$^-$) Barley Lectin cDNAs Introduced into Tobacco.

(a) The 15-amino acid COOH-terminal propeptide of barley lectin [amino acids (aa) 172 through 186] and the corresponding nucleotide sequence [nucleotides (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 that 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).

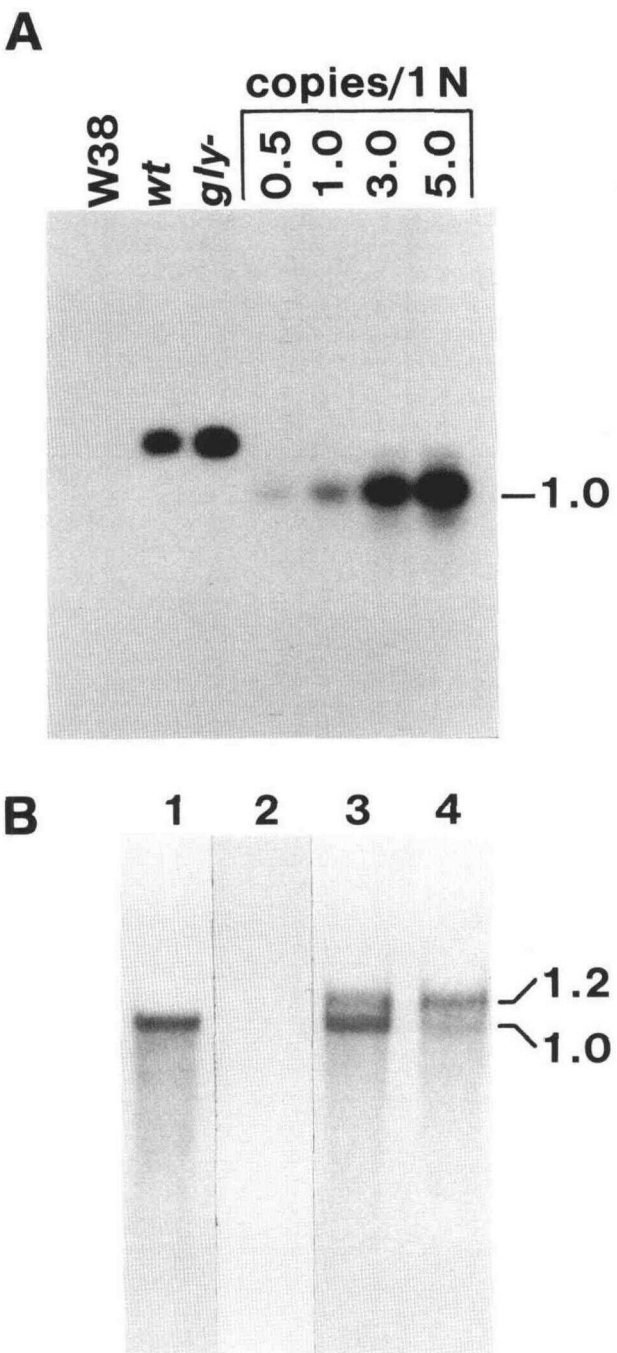
RESULTS

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. As shown in Figure 1b, the preproprotein is composed of a 2.5-kD signal sequence, the 18-kD mature protein, and a 1.5-kD COOH-terminal propeptide. In barley embryos, the proprotein is modified by the addition of a 2-kD high-mannose oligosaccharide side chain to the sole N-linked glycosylation site located within the COOH-terminal propeptide at Asn^{180}-Ser-Thr^{182} (Figure 1a). To investigate further 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 was 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 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 were examined in independent transformants by DNA gel 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 kb to 9.0 kb) and five fragments (18 kb and 2.8 kb to 4.0 kb) were detected in tobacco genomic DNA isolated from *wt* and *gly*⁻ transformants, respectively (data not shown). Gene reconstruction experiments, shown in Figure 2A, were performed with EcoRI-restricted tobacco DNA and purified BLc3 insert titered at 0.5-copy, 1.0-copy, 3.0-copy, 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 were investigated by RNA gel blot analysis. The RNA gel 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 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

Figure 2. Gene Reconstruction Analysis and Accumulation of Steady-State RNA Levels of Barley Lectin in Transgenic Tobacco.

(A) DNA gel blot containing 12 μg 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-copy, 1.0-copy, 3.0-copy, and 5.0-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 kilobases) is shown on the right.

(B) RNA gel blot containing 25 μg 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 sizes of barley lectin mRNA species (in kilobases) are shown on the right.

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 levels 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 bind specifically oligomers of the carbohydrate *N*-acetylglucosamine (GlcNAc). Because the carbohydrate binding site of wheat germ agglutinin (WGA) is composed 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 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 mechanisms of dimerization and carbohydrate binding of WGA and barley lectin are presumably identical.

To determine whether 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. The results are shown in Figure 3. Because 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 after affinity chromatography (Figure 3) indicated that the 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 enzyme-linked immunosorbent assay (ELISA). A range of 800 ng to 2 μg of affinity-purified barley lectin per 1 g of leaf tissue, fresh weight, 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.

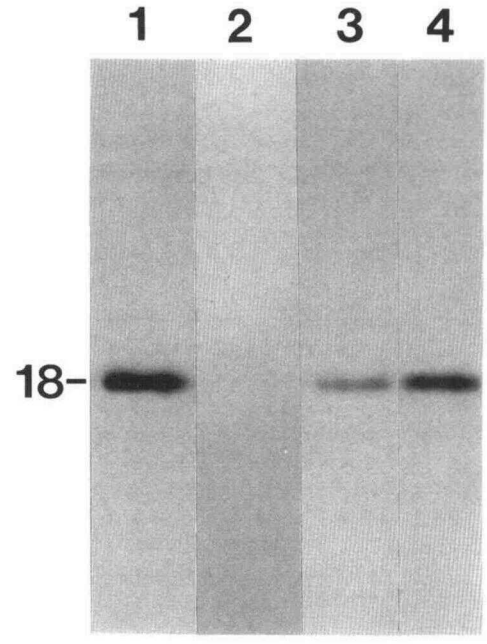


Figure 3. Immunoblot Detection of Mature Barley Lectin in *wt* and *gly*$^-$ tobacco Transformants.

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 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 μg of purified WGA. The molecular mass of mature WGA and barley lectin subunits (in kilodaltons) is shown on the left.

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

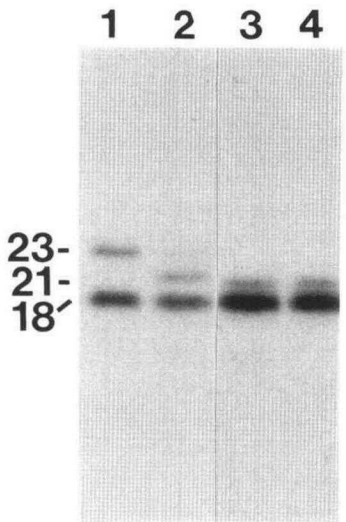


Figure 4. Endo H Digestion of Radiolabeled Barley Lectin Isolated from Transgenic Tobacco.

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 positions and molecular masses (in kilodaltons) of barley lectin *wt* and *gly*$^-$ proproteins (23 kD and 21 kD, respectively) and mature barley lectin (18 kD) are shown on the left.

cultures and pulse labeled for 12 hr in the presence of ^{35}S-trans label. Radiolabeled barley lectin was recovered from tobacco protoplasts by affinity chromatography on immobilized GlcNAc columns. After affinity chromatography, eluant fractions were treated with Endo H, an enzyme that 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 after separation by SDS-PAGE and fluorography, as shown in 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 after treatment with Endo H (lane 2, Figure 4), indicating that the 23-kD polypeptide contained a 2-kD high-mannose glycan. These results imply that the signal sequence had been cleaved and that the synthesis and processing of barley lectin precursors in tobacco were analogous to processing mechanisms in barley. As expected, the *gly*$^-$ barley lectin was synthesized as a 21-kD proprotein (lane 3, Figure 4) that 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_r 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 electron microscopic immunocytochemistry. Figure 5A shows protoplasts prepared from both *wt* and *gly*$^-$ transgenic tobacco plants. 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 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: (1) peroxisomes are very fragile and, consequently, lyse during preparation of vacuoles, thereby liberating catalase into the cell lysate; and (2) 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, as shown in lanes 2 and 4 of 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.

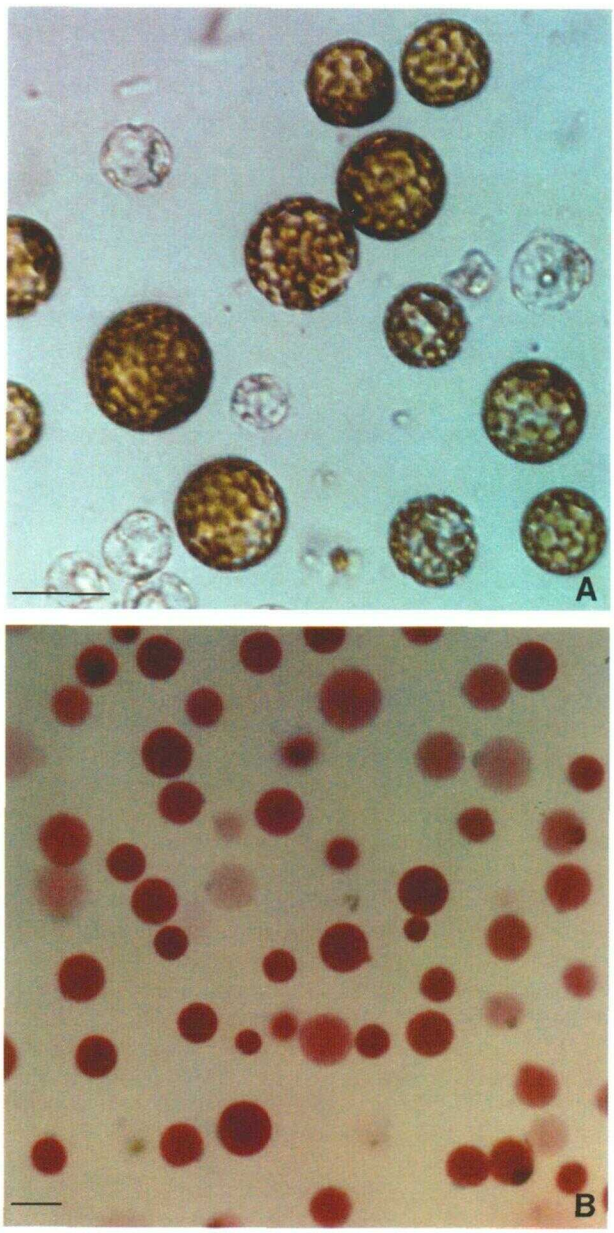


Figure 5. Isolation of Vacuoles from Tobacco Protoplasts Expressing *wt* or *gly*$^-$ Barley Lectin.

(A) Protoplasts were prepared by enzymatic digestion of tobacco leaves collected from axenically cultured transgenic plants. Bar = 10 μm.

(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 μm.

The vacuolar distribution of barley lectin in *wt* and *gly*$^-$ transgenic tobacco leaves 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 hr. 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, Figures 7a and 7b). During the chase period, both the *wt* and *gly*$^-$ radiolabeled proproteins gradually disappeared over time (Figures 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 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) is processed to the mature protein at least 2 times faster than the *wt* 23-kD proprotein ($t_{1/2}$ = 2.0 hr). The disappearance of both *wt* and *gly*$^-$ proproteins displayed linear

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.

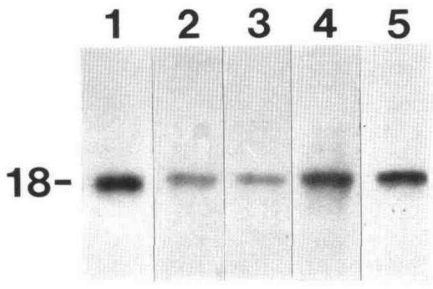


Figure 6. Immunodetection of Mature Barley Lectin in Protoplasts and Vacuoles Isolated from *wt* and *gly*⁻ Transgenic Tobacco Plants.

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 (in kilodaltons) is shown on the left.

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 (Chrispeels, 1983; Tartakoff, 1983). After 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* 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 fewer 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 were 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

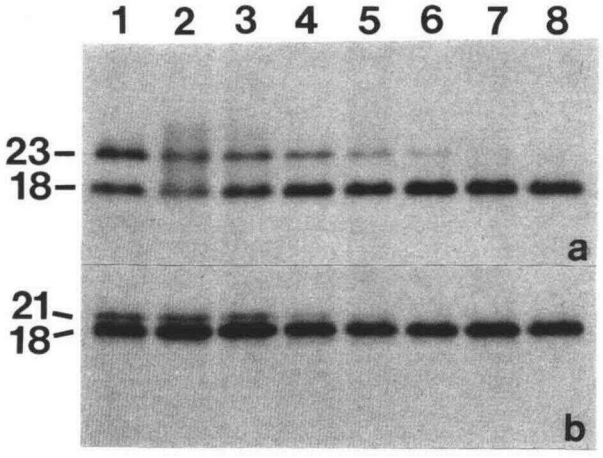


Figure 7. Pulse-Chase Labeling Experiments of Tobacco Protoplasts Expressing *wt* or *gly*⁻ Barley Lectin.

(a) Tobacco protoplasts expressing *wt* barley lectin.
(b) Tobacco protoplasts expressing *gly*⁻ barley lectin.
Protoplasts were pulse labeled for 10 hr and chased for 0 hr, 1 hr, 2 hr, 3 hr, 4 hr, 6 hr, 8 hr, and 10 hr (lanes 1 to 8). Radiolabeled barley lectin was affinity purified from lysed protoplasts, and the eluants were subjected to SDS-PAGE and fluorography. The positions and molecular masses of the *wt* (23 kD) and *gly*⁻ (21 kD) barley lectin proproteins and the 18-kD mature polypeptide are shown on the left.

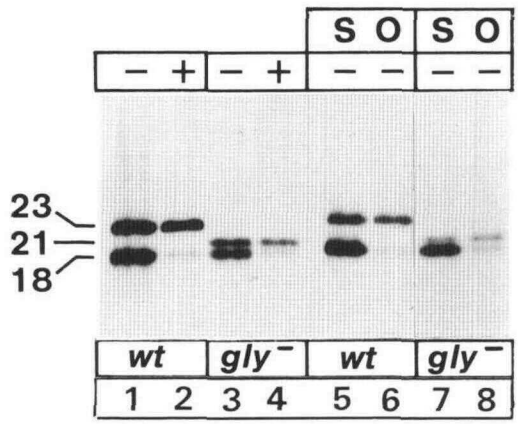


Figure 8. Inhibition of Proteolytic Processing of Barley Lectin Proproteins in the Presence of Monensin.

Tobacco protoplasts expressing *wt* or *gly*⁻ barley lectin were pulse labeled for 12 hr in 0.1% ethanol (−) or 50 μM 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) fractions from the remaining protoplasts were separated by Sepharose 4B chromatography. Radiolabeled barley lectin in subcellular fractions of pulse-labeled tobacco protoplasts was affinity purified and fractionated by SDS-PAGE and treated for fluorography. The positions and molecular masses (in kilodaltons) of barley lectin proproteins and mature polypeptide are shown on the left.

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 were congruent with a shorter half-life for *gly*⁻ proproteins (Figure 7).

DISCUSSION

Barley lectin is a member of a class of vacuolar proteins that 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 indicated 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 is shown to be correctly processed in vegetative tissues of tobacco (Sonnewald et al., 1989). The present study demonstrates the correct processing and stable accumulation of an 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 would 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 pro-ConA apparently plays a direct role in processing and transport of ConA to vacuoles (Faye and Chrispeels, 1987).

Propeptide Glycan Affects the 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 times faster than the glycosylated (*wt*) 23-kD proprotein. Monensin effectively inhibited the post-translational processing of both the *wt* and *gly*⁻ barley 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 pro-ConA 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 pro-ConA (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 modulated 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 relies upon a sequential two-step processing of the proprotein COOH-terminal glycopeptide, as diagramed in Figure 9. Concomitant with the formation of an active lectin dimer, the proprotein assumed a conformation in which the high-mannose glycan sequestered the propeptide from the aqueous environment, thereby masking the availability of the propeptide for processing (Figure 9, top panel). This predicted protein configuration was 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 from the proprotein in a regulated manner. As a consequence, deglycosylation exposed the propeptide to proteases and thereby facilitated further processing and transport of the proprotein (Figure 9, middle panel). Therefore, the deglycosylation of the glycopeptide was 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 was congruous with the involvement of N-linked glycans in the proteolytic processing and stabilization of many mammalian glycoproteins (Olden et al., 1985).

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

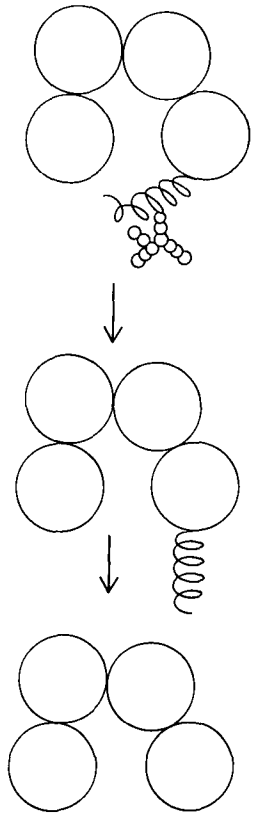


Figure 9. Proposed Cascade of Events Involved in the Post-Translational Processing of Barley Lectin.

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 N-linked glycosylation site (Asn-Ser-Thr) residing within the COOH-terminal propeptide of barley lectin. Structure of high-mannose type glycan was adapted from Montreuil (1984).

DNA polymerase I Klenow fragment as described in Maniatis et al. (1982). After 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 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 a Muta-Gene phagemid in vitro mutagenesis kit (Bio-Rad) 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⁻ Escherichia 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 plâtes (An et al., 1988) containing streptomycin (200 μgl/mL), kanamycin (25 μg/mL), and tetracycline (5 ; gmg gmg/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 co-cultivated with the Agrobacteria for 48 hr on MS104 plates before 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 μg) was restricted with HindIII and fractionated on 1.0% agarose gels before 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-copy, 1.0-copy, 3.0-copy, and 5.0-copy equivalents per tobacco genome (4.8×10^9 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 X-omat AR film (Kodak) 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 μg) 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 col-

umns 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 of 50 mM Tris-acetate, pH 5.0, 100 mM NaCl, 1 mM PMSF. The extracts were clarified by centrifugation at 10,000 rpm 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, San Mateo, CA), 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, 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 were 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 composed of 0.5% cellulase Onozuka R10, 0.25% macerozyme R10 (Yakult Honsha Co., Ltd. Japan), and 0.1% BSA in Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 1.0 μg/mL benzyladenine, 0.1 μg/mL naphthaleneacetic acid, and 0.5 M mannitol (MSA). The yield of protoplasts was quantitated using a hemocytometer counting chamber.

For pulse-labeling experiments, 1×10^5 leaf protoplasts (per well) were incubated in a 24-well Falcon tissue culture plate in 500 μL of MSA medium supplemented with 48 μCi of ^{35}S-Trans label [ICN K & K Laboratories (Plainview, NY) ^{35}S *E. coli* hydrolysate labeling reagent containing ≥70% L-methionine and ≤15% L-cysteine; 1000 Ci/mmol to 1200 Ci/mmol]. 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 hr to 10 hr after pulse-labeling protoplasts as described above. After labeling, protoplasts were pooled and collected by centrifugation at 2,000 rpm for 15 sec at 4°C. The resulting protoplast pellet was suspended in 100 μL of 50 mM Tris-acetate, pH 5.0, 100 mM NaCl and lysed at room temperature for 10 min with gentle agitation after the addition of 100 μL 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 H digestion of radiolabeled barley lectin was performed at 37°C for 23 hr in 50 mM Tris-acetate, pH 5.5, 100 mM NaCl, 1 mM PMSF with 4 milliunits of Endo H immediately after 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 μM 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 before 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 separated from soluble proteins. A total of 600,000 *wt* or *gly*$^-$ protoplasts were pooled and gently homogenized in 200 μL 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 × 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, 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. After collection of protoplasts by centrifugation (see above), the culture medium was recovered from a total of 800,000 protoplasts and contaminating intact protoplasts were removed by gravity filtration through an Isolab (Akron, OH) Quik-Sep column fitted with a paper filter and a Whatman GF/C glass fiber filter (1.2 μm exclusion). Proteins contained in the culture medium were precipitated with ammonium sulfate at 60% saturation at 4°C for at least 2 hr. Precipitated proteins were collected by centrifugation for 10 min at 15,000 rpm. The protein pellet was resuspended in 200 μL 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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