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The Barley Lectin Carboxyl-Terminal Propeptide Is a Vacuolar Protein Sorting Determinant in Plants

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

INTRODUCTION

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

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

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

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

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identified a tetrapeptide Gln-Arg-Pro-Leu (QRPL) to be critical for sorting of the proprotein to the vacuole (Valls et al., 1990).

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

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

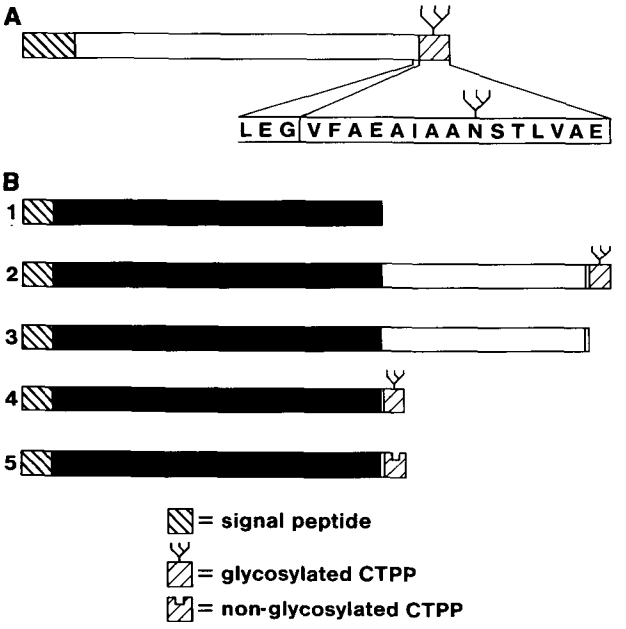

Figure 1. Schematic Representation of proBL/Cuc Chit Fusion Proteins.

(A) The preproprotein of barley lectin consists of a signal sequence (box with dark hatched lines, left), a mature 18-kD subunit (white box, center), and the CTPP (box with light hatched lines, right). The insert represents the 15-amino acid CTPP propeptide (white box with letters). Gly (G) is the last amino acid from the carboxyl terminus of the mature BL preceding the CTPP. Leu (L) and Glu (E) (white box at left of insert) were added by introduction of an XhoI restriction site (see Methods).
(B) Lane 1, the preprotein of Cuc Chit contains a signal sequence (box with dark hatched lines, left) and the mature 28-kD polypeptide (solid black box). Lane 2, Cuc Chit is fused with the 23-kD glycosylated BL proprotein (Cuc Chit-proBL). Lane 3, Cuc Chit is fused with the mature 18-kD BL subunit (Cuc Chit-BL). Lane 4, Cuc Chit is fused with the glycosylated CTPP (Cuc Chit-CTPP). Lane 5, Cuc Chit-CTPP fusion protein that has been modified by site-directed mutagenesis to prevent core glycosylation of the CTPP (Cuc Chit-CTPP[Gly^-]).

RESULTS

Assembly of Cucumber Chitinase Gene Fusions

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

We have demonstrated previously that BL is correctly processed and targeted in transgenic tobacco cells (Wilkins et al., 1990). Deletion of the 15-amino acid CTPP resulted in secretion of BL, indicating that the CTPP is necessary for the proper sorting of this protein to the vacuoles of plant cells (Bednarek et al., 1990). To examine whether the CTPP is necessary and sufficient to redirect a reporter protein to plant vacuoles, a chimeric gene containing the cDNA encoding cucumber chitinase (Cuc Chit) (Metraux et al., 1989) was fused with the region of the BL cDNA encoding the CTPP (Lerner and Raikhel, 1989) (Figure 1B, lane 4). In addition, two gene fusions were constructed to determine whether there are any

additional topogenic signals within the mature BL 18-kD polypeptide sequence necessary for vacuolar protein sorting (Figure 1B, lanes 2 and 3).

Cuc Chit is a protease-resistant 28-kD protein that is secreted into the intercellular space of the cucumber plant in response to viral or pathogen infection (Metraux et al., 1989). No significant homology is found in a comparison of the DNA and deduced amino acid sequences of Cuc Chit and the intracellular basic chitinase isoforms from tobacco and bean (Metraux et al., 1989). In addition, polyclonal anti-Cuc Chit antisera do not cross-react with the chitinases from tobacco (J. Ryals, personal communication). Endonuclease restriction sites were introduced by site-directed mutagenesis (see Methods) into cDNA genes encoding Cuc Chit and proBL to facilitate subcloning of proBL restriction fragments onto the 3′ end of the Cuc Chit open reading frame. Figure 1B is a schematic representation of the proteins encoded by the proBL/Cuc Chit restriction fragment gene fusions. Three Cuc Chit gene fusions were constructed containing the following sequences: (1) the region encoding the BL proprotein (Figure 1B, lane 2), (2) the region encoding only the mature 18-kD subunit (Figure 1B, lane 3), and (3) the CTPP coding region (Figure 1B, lane 4). Although it has been demonstrated that the last carboxyl-terminal amino acid of the mature lectin subunit is a glycine residue (Gly^{171}) (Wright et al., 1984), the exact site within proBL at which the CTPP is cleaved has yet to be defined. For this reason, we have engineered the 3′ end of the Cuc Chit open reading frame to mimic the last two carboxyl-terminal amino acids of the mature 18-kD BL subunit. The Cuc Chit-CTPP fusion gene was assembled (see Methods) such that the carboxyl-terminal amino acids of Cuc Chit preceding the CTPP were an acidic amino acid (Glu) followed by a glycine residue.

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

Analysis of Cuc Chit Gene Fusions in Transformed Tobacco Cells

To facilitate a rapid analysis of the proBL/Cuc Chit fusion proteins, Cuc Chit/pGA643 constructs were introduced into tobacco suspension-cell protoplasts by polyethylene glycol-mediated DNA uptake (Bednarek et al., 1990), and the protoplasts were labeled in the presence of a mixture of ^{35}S-labeled methionine and cysteine (^{35}S-Met/Cys) for 14 hr. Cuc Chit and Cuc Chit fusion proteins were purified from protein extracts of the radiolabeled protoplasts and incubation media by immunoprecipitation with polyclonal antisera directed against Cuc Chit and analyzed by SDS-PAGE and fluorography. As shown in Figure 2, Cuc Chit and Cuc Chit-BL were synthesized as single polypeptides of M_r 28,000 and M_r 46,000, respectively, and secreted from the protoplasts into the incubation media (Figure 2, lanes 1 and 2), indicating that these proteins were properly translocated into the tobacco secretory system and secreted. The labeled polypeptide with an M_r of 46,000 was completely secreted from the tobacco protoplast during a 10-hr chase with unlabeled methionine and cysteine (Met/Cys) and accumulated in the media (data not shown). In tobacco protoplasts transformed with Cuc Chit-proBL, two polypeptides of M_r 51,000 (Figure 1B, lane 2) and M_r 46,000 were detected intracellularly (Figure 2, lane 3). These results imply that the Cuc Chit-proBL fusion protein was modified by glycosylation (M_r 51,000) and subsequently processed by removal of the glycopropeptide to the polypeptide with an M_r of 46,000. Likewise, in tobacco protoplasts transformed with Cuc Chit-CTPP, two polypeptides of M_r 33,000 (corresponding to the predicted molecular mass of glycosylated Cuc Chit-CTPP) and M_r 28,000 were detected intracellularly (Figure 2, lane 4). The

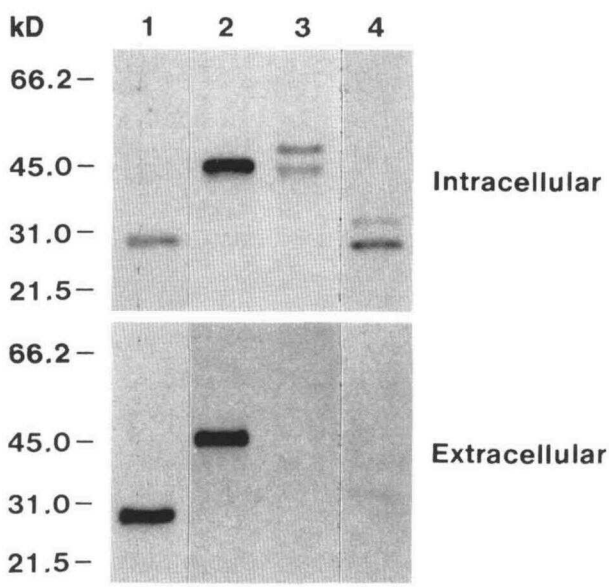

Figure 2. Analysis of Transiently Expressed Cuc Chit Fusion Proteins in Tobacco Protoplasts.

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

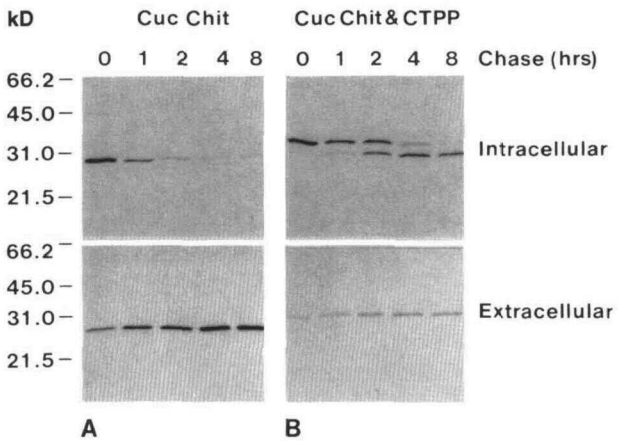

Figure 3. Pulse-Chase Labeling Experiments of Tobacco Protoplasts Expressing Cuc Chit and Cuc Chit-CTPP Fusion Proteins.

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

presence of the intracellular polypeptide with an M_r of 28,000 suggests that Cuc Chit-CTPP was properly sorted and processed in tobacco cells. In addition, a single polypeptide (M_r 33,000) corresponding to Cuc Chit-CTPP proprotein was detected in the incubation media of tobacco protoplasts transformed with Cuc Chit-CTPP (Figure 2, lane 4). A very low level of a protein (M_r 51,000) was secreted from tobacco protoplasts expressing Cuc Chit-proBL. The secreted radiolabeled polypeptide with an M_r of 51,000 was discernible only after a very long exposure (>4 weeks) of the fluorogram shown in Figure 2 (data not shown).

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

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

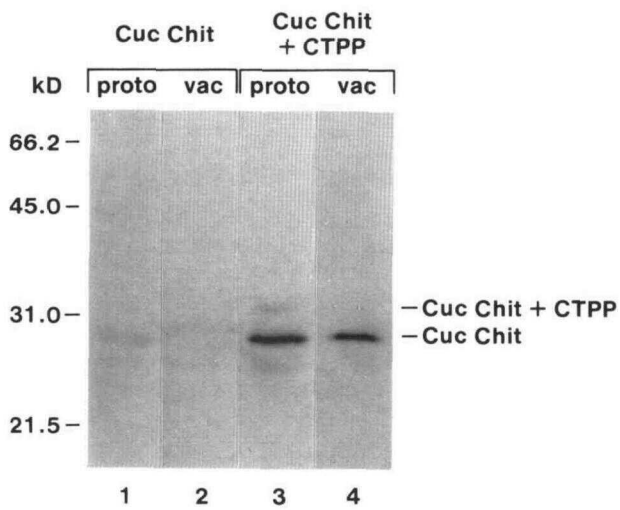

Figure 4. Localization of the Processed Form of the Cuc Chit-CTPP Fusion Protein in the Vacuoles of Cuc Chit-CTPP Transgenic Tobacco Protoplasts.

Total protein from protoplasts (proto) and isolated vacuoles (vac) were separated by electrophoresis on a 12.5% SDS-polyacrylamide gel and electroblotted onto Immobilon-P membrane. Immunodetection of Cuc Chit was performed with anti-Cuc Chit antisera as described in Methods. Equal amounts of soluble vacuole proteins in the protoplast (lanes 1 and 3) and vacuole (lanes 2 and 4) fractions, relative to α-mannosidase activity, were loaded per lane. The sizes of molecular mass standards are shown on the left in kilodaltons.

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

We have previously shown that the 23-kD proprotein and the mature 18-kD subunit of BL are localized in the microsomal fraction and vacuoles of transgenic tobacco cells, respectively (Wilkins et al., 1990). The subcellular localizations of Cuc Chit and Cuc Chit-CTPP were examined by organelle isolation, as shown in Figure 4, and by electron microscopic immunocytochemistry, as shown in Figure 5. Protoplasts for vacuole isolation were prepared from the same transgenic plants used in the pulse-chase experiments (Figure 3) to ensure similar levels of Cuc Chit and Cuc Chit-CTPP fusion protein expression, and vacuoles were isolated as described in Methods. Activities of enzymes specific for the cytosol (glucose-6-phosphate dehydrogenase, EC 1.1.1.49) (Simcox et al., 1977), the ER (NADH-cytochrome *c* reductase, EC 1.6.99.3) (Lord, 1983), and the vacuole (α-mannosidase, EC 3.2.1.24) (Boller and Kende, 1979) were compared in crude protoplast and vacuole lysates. Vacuole fractions from Cuc Chit and Cuc Chit-CTPP plants contained $\leq$10% NADH-cytochrome *c* reductase and $\leq$5% glucose-6-phosphate dehydrogenase, relative to total protoplast-associated activity. The subcellular distribution of Cuc Chit and Cuc Chit-CTPP proteins was examined by immunoblot analysis of the protoplast and vacuole lysates using Cuc Chit polyclonal sera. Gels were loaded such that each lane

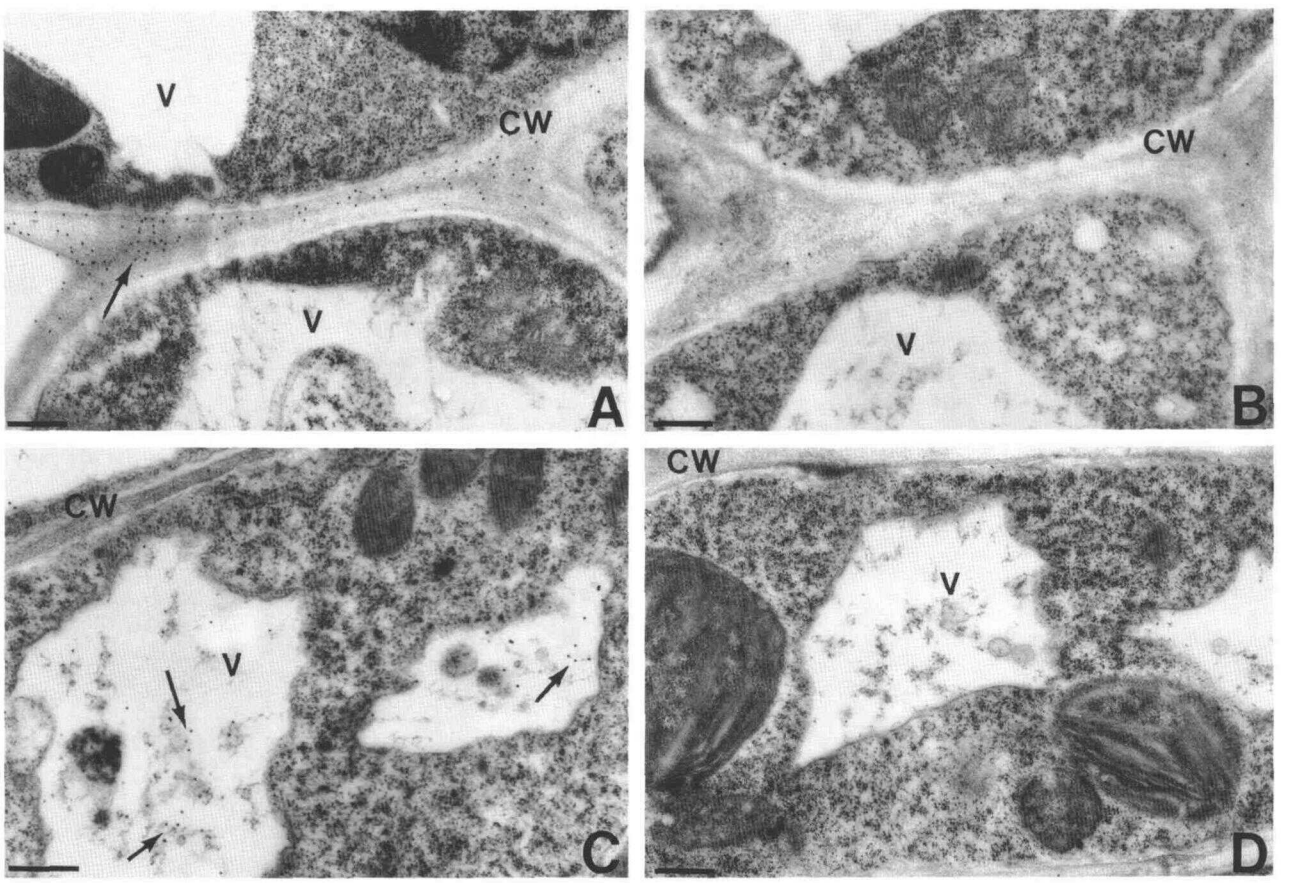

Figure 5. Immunocytochemical Localization of Cuc Chit and Cuc Chit-CTPP Fusion in Transgenic Tobacco Cells.

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

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

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

contained the same amount of total vacuolar protein with respect to α-mannosidase activity. The processed form of Cuc Chit-CTPP proprotein (M_r 28,000) was detected only in the vacuole fraction from Cuc Chit-CTPP protoplasts, indicating that the CTPP is sufficient for redirection of Cuc Chit to the vacuoles of plants (Figure 4, lane 4).

Localization of Cuc Chit in transgenic tobacco plants was also analyzed by electron microscopic immunocytochemistry. Thin sections of transgenic tobacco leaves expressing Cuc Chit and Cuc Chit-CTPP were treated with Cuc Chit antiserum. Antibody binding was visualized with 15-nm-diameter colloidal gold linked to protein A. Cuc Chit was localized in the cell wall and middle lamella of tobacco cells expressing Cuc Chit (Figure 5A), whereas colloidal gold labeling was readily discernible in the vacuoles of tobacco cells expressing Cuc Chit-CTPP (Figure 5C). A very low level of labeling was also detected in the cell wall of these cells. No specific labeling was detected in parallel experiments using nonimmune sera as the primary antibody (Figures 5B and 5D).

Glycosylation of Cuc Chit-CTPP Fusion Protein

In barley embryos and transgenic tobacco, proBL is modified by the addition of a high-mannose oligosaccharide with a molecular mass of approximately 2 kD to the CTPP (Lerner and Raikhel, 1989; Wilkins et al., 1990). The mobility of Cuc Chit-CTPP proprotein (M_r 33,000) on SDS-polyacrylamide gels suggested that the CTPP was similarly modified in the fusion protein. To examine whether the CTPP of Cuc Chit-CTPP was glycosylated, immunoprecipitated proteins from radiolabeled Cuc Chit-CTPP protoplasts and incubation media were digested with endo-β-*N*-acetylglucosamine H (endo H), an enzyme that specifically cleaves high-mannose oligosaccharide side chains. The majority of intracellular Cuc Chit-CTPP (M_r 33,000) was converted to a protein with an M_r of 30,000 by treatment with endo H, as shown in Figure 6B. Interestingly, the extracellular polypeptide (M_r 33,000) was insensitive to digestion with endo H (Figure 6B). We were unable to establish whether the endo H-resistant oligosaccharide side chain of Cuc Chit-CTPP proprotein was the result of modification of the high-mannose glycan to a complex type (data not shown). ^{35}S-labeled proBL was treated with endo H as a positive control for endo H activity. As previously described (Wilkins et al., 1990), proBL (23 kD) was deglycosylated by endo H treatment to a 21-kD polypeptide (data not shown).

We have examined the possibility that secretion of Cuc Chit-CTPP (M_r 33,000) resulted from the presence of the endo H-resistant glycan on the proprotein. Protoplasts expressing Cuc Chit-CTPP were labeled for 4 hr with ^{35}S-Met/Cys in the presence of 25 μM tunicamycin to block *N*-linked glycosylation. Labeled proteins were chased for an additional 10 hr with excess unlabeled Met/Cys, as

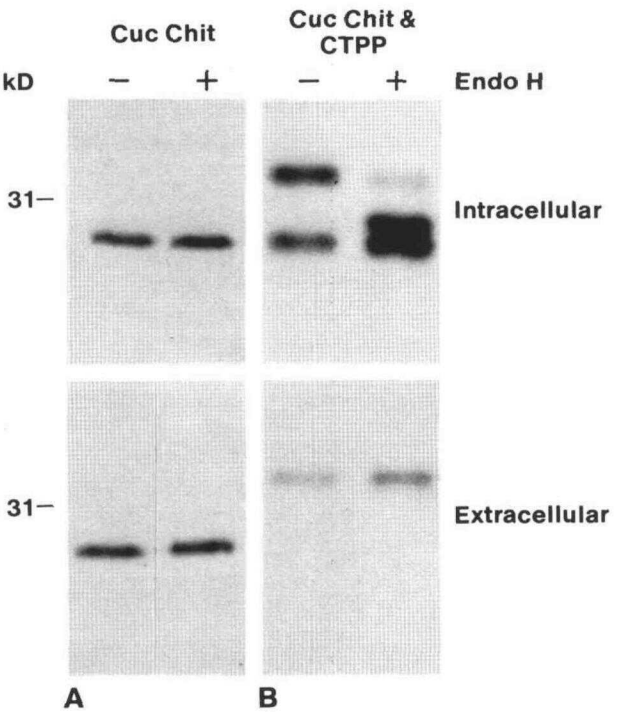

Figure 6. Endo H Digestion of Radiolabeled Cuc Chit and Cuc Chit-CTPP Fusion Protein.

(A) and **(B)** Radiolabeled proteins were immunopurified from the intracellular and extracellular fractions of tobacco protoplasts expressing Cuc Chit **(A)** and Cuc Chit-CTPP **(B)**. Duplicate samples were incubated at 37°C for 18 hr in the absence (−) or presence (+) of endo H before analysis by SDS-PAGE and fluorography. Molecular mass markers are indicated on the left in kilodaltons.

shown in Figure 7. Similar experiments were performed with a mutant form of Cuc Chit-CTPP in which the CTPP glycosylation site (Asn-Ser-Thr) was altered by site-directed mutagenesis (Gly-Ser-Thr) to prevent attachment of the *N*-linked glycan (see Figure 1B, lane 5, for a schematic representation of the Cuc Chit-CTPP[Gly$^-$] fusion protein) (data not shown). Levels of Cuc Chit were quantitated in intracellular and extracellular fractions from tunicamycin-treated Cuc Chit-CTPP protoplasts and tobacco protoplasts expressing Cuc Chit-CTPP[Gly$^-$] as described above. Inhibition of Cuc Chit-CTPP glycosylation by either method only slightly increased the level (80% to 85%) of the Cuc Chit-CTPP proprotein that was processed and retained intracellularly.

Stability of Cuc Chit in the Vacuole and Media

To examine the stability of Cuc Chit and Cuc Chit-CTPP protein in the vacuole and media, protoplasts were pulse

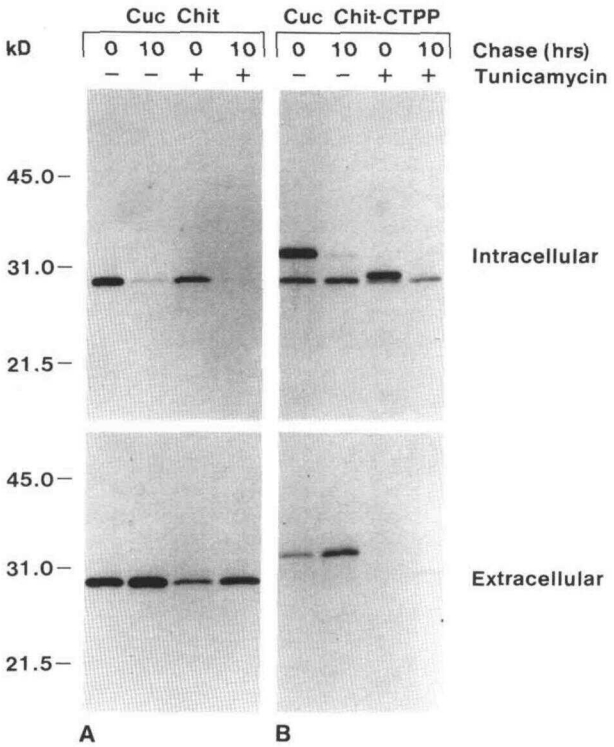

Figure 7. Effect of Core Glycosylation Inhibition on Sorting of the Cuc Chit-CTPP Proprotein to the Vacuole.

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

labeled for 2.5 hr with ^{35}S-Met/Cys and radiolabeled proteins were chased with unlabeled Met/Cys for an additional 10 hr to deplete the intracellular pool of ^{35}S-labeled Cuc Chit and Cuc Chit-CTPP proprotein. Intracellular and extracellular proteins were purified by immunoprecipitation at 2- to 4-hr intervals during an additional 20-hr chase period. The rate of Cuc Chit turnover in the vacuole and media was quantitated by densitometry. The polypeptide with an M_r of 28,000 was degraded with a $t_{1/2}$ of 5 hr in the vacuole. No degradation of either Cuc Chit (M_r 28,000) or Cuc Chit-CTPP (M_r 33,000) in the media was observed.

DISCUSSION

Sorting of vacuolar and lysosomal proteins from other secretory proteins requires specific targeting information contained within the molecular structure of these polypeptides. Chimeric proteins containing a secreted protein and various regions of a vacuolar protein have been used to characterize vacuolar targeting information in yeast (Johnson et al., 1987; Klionsky et al., 1988). Using a similar approach, we have demonstrated that the vacuolar sorting determinant of the plant protein BL is contained within a 15-amino acid CTPP. BL is initially synthesized as a glycosylated proprotein and is subsequently processed before or concomitant with deposition of the protein in the vacuole by removal of the glycosylated CTPP. Similarly, both Cuc Chit-CTPP and Cuc Chit-proBL fusion proteins were initially synthesized as proproteins and processed to their mature form by the intracellular removal of the CTPP. We have further demonstrated by organelle purification and electron microscopy immunocytochemical localization that the M_r 28,000 processed form of Cuc Chit-CTPP is localized in the vacuoles of tobacco cells expressing Cuc Chit-CTPP. Thus, the proBL CTPP was sufficient to redirect a secreted protein, Cuc Chit, to the plant vacuole.

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

Sorting Efficiency

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

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

fusion or deletions near this region containing the tetrapeptide QRPL, which is critical for CPY sorting, may interfere with the structural context in which the signal is presented and may result in missorting of the CPY or the CPY-invertase fusion protein.

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

Analysis and Comparison of Plant Vacuolar Sorting Determinants

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

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

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

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

Conclusions

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

METHODS

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

Construction of Cuc Chit Gene Fusions

pSCU1 contained a Cuc Chit gene (Metraux et al., 1989) in which the putative Cuc Chit signal sequence coding region (amino acids 1 to 26) had been replaced with the signal peptide DNA sequence from the basic tobacco chitinase (amino acids 1 to 26) (Shinshi et al., 1987, 1990). The restriction fragment containing the Cuc

Chit insert from pSCU1 was subcloned into pUC118 (Vieira and Messing, 1987). SalI and XbaI restriction sites were inserted in the 5′ untranslated region of Cuc Chit by site-directed mutagenesis (Kunkel et al., 1987). An additional XhoI site was created by site-directed mutagenesis preceding the codon for Gly^{289} of the Cuc Chit deduced amino acid sequence (Metraux et al., 1989). This construct will be called Cuc Chit (Figure 1B, lane 1).

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

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

Transient Gene Expression in Tobacco Suspension Protoplasts

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

To examine expression, transformed protoplasts were incubated for 14 hr in the dark at room temperature with gentle shaking in the presence of 100 μCi of ^{35}S protein-labeling mixture (^{35}S-Met/Cys) (specific activity, 1000 Ci/mmol to 1100 Ci/mmol) (Du Pont-New England Nuclear, Boston, MA). Labeled proteins were chased for an additional 10 hr with an excess of unlabeled methionine and cysteine (final concentration of 15 and 7.5 mM, respectively). Protoplasts and incubation media were transferred to 1.5-mL microcentrifuge tubes and separated by brief centrifugation (15 to 20 sec) at 800*g*. The protoplast pellets were lysed in 500 μL of TNET250 (25 mM Tris-HCl, pH 7.5, 250 mM NaCl, 5 mM EDTA, 1% Triton X-100 [v/v]) (Firestone and Winguth, 1990) and cleared of insoluble debris by centrifugation at 16,000*g* for 5 min at 4°C. The extracellular protein fraction was prepared from the filtered incubation media as described in Bednarek et al. (1990) with the exception that 50 mg of BSA was added as nonspecific "carrier" protein. The culture medium/BSA protein precipitates were resuspended in 500 μL of TNET250. For immunoprecipitation, 100 μL of 50 mg/mL BSA was added to the protoplast and media extracts.

Plant Transformation

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

Isolation and Radiolabeling of Transformed Tobacco Leaf Protoplasts

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

Vacuole Isolation

Protoplasts for vacuole isolation were prepared as described above. Vacuoles were released from the protoplasts by a combination of osmotic and thermal shock. Viable protoplasts (1×10^7) were chilled on ice for 30 min and then pelleted at 50*g* for 10 min at 4°C. Protoplast were gently lysed in lysis buffer (0.2 M sorbitol, 10% [w/v] Ficoll 400, 10 mM Hepes-KOH, pH 7.5, 10 μg/mL neutral red) and preheated to 45°C. Vacuoles were purified by flotation on a discontinuous Ficoll density gradient. The protoplast lysate was overlaid with two steps containing 5% (w/v) Ficoll 400 in 0.6 M betaine, 10 mM Hepes-KOH, pH 7.5, and 0.6 M betaine, 10 mM Hepes-KOH, pH 7.5; the gradients were centrifuged in a swinging bucket rotor at 5000*g* for 30 min at 4°C. Vacuoles were recovered from the 0%/5% (w/v) Ficoll 400 interface, quantitated using a hemocytometer, and gently lysed by osmotic shock. The vacuole suspension was diluted with 5 volumes of 10 mM Hepes-KOH, pH 7.5, and incubated at room temperature for 10 min. Membranes and unbroken vacuoles were cleared from the lysate by centrifugation at 100,000*g* for 30 min at 4°C. Soluble proteins were concentrated by ammonium sulfate (70% saturated at 20°C) and resuspended in 10 mM Hepes-KOH, pH 7.5, 0.5% (v/v) Triton X-100. For subcellular marker enzyme assays, extracts representing total protoplast proteins were prepared. Protoplasts were lysed in 10 mM Hepes-KOH, pH 7.5, 0.5% (v/v) Triton X-100 and cleared of insoluble material by centrifugation at 16,000*g* for 10 min at 4°C.

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

Gels were electroblotted onto Immobilon-P membrane (Millipore Corp., Bedford, MA) (Towbin et al., 1979), and the membranes were blocked for 2 hr with TBS (20 mM Tris-HCl, pH 7.4, 150 mM NaCl) containing 3% (w/v) gelatin and 1% (w/v) BSA. The membranes were incubated for 1.5 hr with anti-Cuc Chit antiserum diluted 1:1250 in TBS containing 1% (w/v) BSA and 0.05% (v/v) Tween-20. After washing in TBS-0.1% (v/v) Tween-20, membranes were incubated for 1 hr with goat anti-rabbit antibody conjugated to alkaline phosphatase (Kirkegaard and Perry Labs Inc., Gaithersburg, MD) diluted 1:5000 in TBS containing 1.0% (w/v) BSA and 0.05% (v/v) Tween-20. Secondary antibody binding was visualized as described by Blake et al. (1984).

Marker Enzyme Assays

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

Immunoprecipitation and Analysis of Immunoprecipitated Proteins

Cuc Chit and Cuc Chit fusion proteins were purified by immunoprecipitation. To remove nonspecifically binding proteins, ^{35}S-labeled protoplast and media extracts were treated with 25 μL of nonimmune rabbit sera for 30 min at room temperature. Nonspecific protein immunocomplexes were reacted with fixed *Staphylococcus aureus* for 30 min at room temperature and removed by centrifugation at 16,000*g* for 5 min. Two microliters of anti-Cuc Chit antiserum was added to the cleared extracts and incubated at room temperature for 15 min. Immunocomplexes were collected on protein A-Sepharose CL-4B beads (Pharmacia, Piscataway, NJ) for 15 min at room temperature and washed three times with TNET250. To reduce nonspecific background further, immunocomplexes were released from the protein A-Sepharose CL-4B beads by detergent solubilization with 1.0% SDS, as described previously (Firestone and Winguth, 1990). The solubilized fraction was diluted in 1200 μL of TNET250 buffer with 0.5 mg of BSA and 0.6 μL of anti-Cuc Chit antiserum and incubated at room temperature for 15 min with continuous mixing. Immunocomplexes were collected on protein A-Sepharose CL-4B beads washed once with TNET250 and once in nondetergent washing buffer (10 mM Tris-HCL pH 7.5, 5 mM EDTA). Bound proteins were released by heating at 95°C for 5 min in 30 μL of SDS-PAGE sample buffer. Samples were analyzed by SDS-PAGE on 12.5% polyacrylamide gels (either 3- or 9-cm running gels) and visualized by fluorography, as described previously (Mansfield et al., 1988). For treatment with endo H, immunopurified proteins were released from protein A-Sepharose CL-4B as described above. Samples were diluted fivefold in double-distilled H_2O and adjusted to 250 mM NaOAc pH 5.5, 1 mM phenylmethylsulfonyl fluoride. The samples were incubated for 18 hr at 37°C in the presence of 5 mU of endo H (Calbiochem Corp., La Jolla, CA), precipitated with ice-cold acetone (final concentration 70%), and resuspended in 50 μL of SDS-PAGE sample buffer. As a positive control for endo H activity, affinity-purified proBL (Wilkins et al., 1990) was resuspended in 30 μL of SDS-PAGE sample buffer and treated as described above. Samples were analyzed on 12.5% SDS-polyacrylamide gels (9-cm running gel), and radiolabeled proteins were visualized by fluorography (Mansfield et al., 1988). Tunicamycin experiments were essentially performed as described in Mansfield et al. (1988), with the exception that the final concentration of tunicamycin was adjusted to 20 μg/mL.

Immunocytochemistry

All procedures were carried out at room temperature. Small pieces of leaf tissues from transgenic tobacco plants were fixed in the mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 10 mM sodium-phosphate buffer (pH 7.2) with 0.1 M sucrose and vacuum infiltrated for 2 hr. After fixation, the tissue was washed in the same buffer with 0.5 M sucrose three times, 10 min each. The tissue was postfixed in 1% OsO_4 in the same buffer with 0.05 M sucrose for 1 hr and then rinsed in distilled water three times, 5 min each. Following dehydration in an ethanol series, the tissue was embedded in London Resin White acrylic resin (Polysciences, Warrington, PA) and polymerized at 60°C under vacuum overnight. Thin sections were prepared on an Ultracut E microtome (Reichert-Jung, Vienna, Austria) and mounted on formvar-coated nickel grids (Polysciences, Warrington, PA). Immunocytochemistry was performed essentially as described by Herman and Melroy (1990). The primary antibody (rabbit anti-chitinase antiserum) was diluted 1 to 20, and control sections were incubated with nonimmune serum diluted similarly. Protein A-colloidal gold (EY Lab Inc., San Mateo, CA) was diluted 1 to 50. Thin sections were examined on a JEOL 100CXII transmission microscope (Tokyo, Japan).

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REFERENCES

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

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

Blake, M.S., Johnston, K.H., Russell-Jones, G.J., and Gotschlich, E.C. (1984). A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on Western blots. Anal. Biochem. **136,** 175–179.

Blobel, G. (1980). Intracellular protein topogenesis. Proc. Natl. Acad. Sci. USA **77,** 1496–1500.

Boller, T., and Kende, H. (1979). Hydrolytic enzymes in the central vacuole of plant cells. Plant Physiol. **63,** 1123–1132.

Boller, T., and Wiemken, A. (1986). Dynamics of vacuole compartmentation. Annu. Rev. Plant Physiol. **37,** 137–164.

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

Denecke, J., Botterman, J., and Deblaere, R. (1990). Protein secretion in plant cells can occur via a default pathway. Plant Cell **2,** 51–59.

Firestone, G.L., and Winguth, S.D. (1990). Immunoprecipitation of proteins. Methods Enzymol. **182,** 688–700.

Herman, E.M., and Melroy, D.L. (1990). Electron microscopic immunocytochemistry in plant molecular biology. Plant Mol. Biol. Manual **B13,** 1–24.

Hunt, D.C., and Chrispeels, M.J. (1991). The signal peptide of a vacuolar protein is necessary and sufficient for the efficient secretion of a cytosolic protein. Plant Physiol. **96,** 18–25.

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

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

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

Kunkel, T.A., Roberts, J.D., and Zakour, R.A. (1987). Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154,** 367–382.

Lerner, D.R., and Raikhel, N.V. (1989). Cloning and characterization of root-specific barley lectin. Plant Physiol. **91,** 124–129.

Linthorst, J.H.M., van Loon, L.C., van Rossum, C.M.A., Mayer, A., Bol, J.F., van Roekel, C., Meulenhoff, E.J.S., and Cornelissen, B.J.C. (1990). Analysis of acidic and basic chitinases from tobacco and petunia and their constitutive expression in transgenic tobacco. Mol. Plant-Microbe Interact. **3,** 252–258.

Lord, J.M. (1983). Endoplasmic reticulum and ribosomes. In Isolation of Membranes and Organelles from Plant Cells, J.L. Hall and A.L. Moore, eds (New York: Academic Press), pp. 119–134.

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

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

Metraux, J.P., Burkhart, W., Moyer, M., Dincher, S., Middlesteadt, W., Williams, S., Payne, G., Carnes, M., and Ryals, J. (1989). Isolation of a complementary DNA encoding a chitinase with structural homology to a bifunctional lysozyme/chitinase. Proc. Natl. Acad. Sci. USA **86,** 896–900.

Mishkind, M.L., Palevitz, B.A., Raikhel, N.V., and Keegstra, K. (1983). Localization of wheat germ agglutinin-like lectins in various species of the Gramineae. Science **220,** 1290–1292.

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

Neale, A.D., Wahleithner, J.A., Lund, M., Bonnett, H.T., Kelly, A., Meeks-Wagner, D.R., Peacock, W.J., and Dennis, E.S. (1990). Chitinase, β-1,3-glucanase, osmotin, and extensin are expressed in tobacco explants during flower formation. Plant Cell **2,** 673–684.

Neuhaus, J.-M., Sticher, L., Meins, F., Jr., and Boller, T. A short C-terminal sequence is necessary and sufficient for the targeting of chitinases to the plant vacuole. Proc. Natl. Acad. Sci. USA, in press.

Rothman, J.E. (1987). Protein sorting by selective retention in the endoplasmic reticulum and Golgi stack. Cell **50,** 521–522.

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

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

Sanger, F., Nicklen, S., and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **56,** 5463–5467.

Shinshi, H., Mohnen, D., and Meins, F., Jr. (1987). Regulation of a plant pathogenesis-related enzyme: Inhibition of chitinase and chitinase mRNA accumulation in cultured tobacco tissues by auxin and cytokinin. Proc. Natl. Acad. Sci. USA **84,** 89–93.

Shinshi, H., Wenzler, H., Neuhaus, J.-M., Felix, G., Hofsteenge, J., and Meins, F., Jr. (1988). Evidence for N- and C-terminal processing of a plant defense-related enzyme: Primary structure of tobacco prepro-β-1,3-glucanase. Proc. Natl. Acad. Sci. USA **85,** 5541–5545.

Shinshi, H., Neuhaus, J.-M., Ryals, J., and Meins, F., Jr. (1990). Structure of a tobacco endochitinase gene: Evidence that different chitinase genes can arise by transposition of sequences encoding a cysteine-rich domain. Plant Mol. Biol. **14,** 357–368.

Simcox, P.D., Reid, E.E., Canvin, D.T., and Dennis, D.T. (1977). Enzymes of the glycolytic and pentose phosphate pathways in proplastids from the developing endosperm of *Ricinus communis* L. Plant Physiol. **59,** 1128–1132.

Smith, J., and Raikhel, N.V. (1989). Nucleotide sequences of cDNA clones encoding wheat germ agglutinin. Plant Mol. Biol. **180,** 601–603.

Tague, B.W., and Chrispeels, M.J. (1987). The plant vacuolar protein, phytohemagglutinin, is transported to the vacuole of transgenic yeast. J. Cell Biol. **105,** 1971–1979.

Tague, B.W., Dickinson, C.D., and Chrispeels, M.J. (1990). A short domain of the plant vacuolar protein phytohemagglutinin targets invertase to the yeast vacuole. Plant Cell **2,** 533–546.

Towbin, H., Staehelin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA **76,** 4350–4354.

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

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

Van den Bulcke, M., Bauw, G., Castresana, C., Van Montagu, M., and Vandekerckhove, J. (1989). Characterization of vacuolar and extracellular β(1,3)-glucanases of tobacco: Evidence for a strictly compartmentalized plant defense system. Proc. Natl. Acad. Sci. USA **86,** 2673–2677.

Vieira, J., and Messing, J. (1987). Production of single-stranded plasmid DNA. Methods Enzymol. **153,** 3–11.

von Figura, K., and Hasilik, A. (1986). Lysosomal enzymes and their receptors. Annu. Rev. Biochem. **55,** 167–193.

Wieland, F.T., Gleason, M.L., Serafini, T.A., and Rothman, J.E. (1987). The rate of bulk flow from the endoplasmic reticulum to the cell surface. Cell **50,** 289–300.

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

Wright, C.S., Gavilanes, F., and Peterson, D.L. (1984). Primary structure of wheat germ agglutinin isolectin 2. Peptide order deduced from x-ray structure. Biochemistry **23,** 280–287.