Colocalization of Barley Lectin and Sporamin in Vacuoles of Transgenic Tobacco Plants[1]

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Various targeting motifs have been identified for plant proteins delivered to the vacuole. For barley (*Hordeum vulgare*) lectin, a typical Gramineae lectin and defense-related protein, the vacuolar information is contained in a carboxyl-terminal propeptide. In contrast, the vacuolar targeting information of sporamin, a storage protein from the tuberous roots of the sweet potato (*Ipomoea batatas*), is encoded in an amino-terminal propeptide. Both proteins were expressed simultaneously in transgenic tobacco plants to enable analysis of their posttranslational processing and subcellular localization by pulse-chase labeling and electron-microscopic immunocytochemical methods. The pulse-chase experiments demonstrated that processing and delivery to the vacuole are not impaired by the simultaneous expression of barley lectin and sporamin. Both proteins were targeted quantitatively to the vacuole, indicating that the carboxyl-terminal and amino-terminal propeptides are equally recognized by the vacuolar protein-sorting machinery. Double-labeling experiments showed that barley lectin and sporamin accumulate in the same vacuole of transgenic tobacco (*Nicotiana tabacum*) leaf and root cells.

Vacuoles are part of the endomembrane system that includes the ER, the Golgi, the TGN, and transitional vesicles mediating transport between the multiple compartments of the endomembrane system. The plant vacuole is a multifunctional organelle essential for numerous functions vital to cellular homeostasis and important for regulatory functions in plant cell growth and development (Boller and Wiemken, 1986). To perform different functions, vacuoles accumulate and eventually release inorganic ions, metabolic intermediates, and secondary plant products; and they contain a complement of acid hydrolases (Herman et al., 1981). Other vacuolar proteins are synthesized at certain stages of plant development and some of them, such as lectins (Chrispeels and Raikhel, 1991) or protease inhibitors (Nelson and Ryan, 1980), are involved in plant defense functions. A different set of hydrolytic enzymes accumulates in plant cells upon pathogen attack, and it has been shown that the most abundant isoforms, the basic 1,3-β-glucanases (Van den Bulcke et al., 1989) and chitinases (Bol et al., 1990), are also localized in the vacuole. Furthermore, the vacuoles can accumulate storage proteins and differentiate to form so-called protein storage vacuoles in vegetative storage tissue such as sweet potato (*Ipomoea batatas*) tubers (Hattori et al., 1985) or as seeds from bean (*Phaseolus vulgaris*) (Bollini and Chrispeels, 1978).

Most of the soluble and membrane-bound secretory proteins enter the secretory pathway by cotranslational insertion into the ER compartment (Chrispeels, 1991). From there, secretory proteins traverse the Golgi and the TGN and are transported to the cell surface via the constitutive pathway if no further sorting signal is present (Hunt and Chrispeels, 1991; Chrispeels, 1991). Special information provided by the protein is required for diversion of the secretory proteins to the vacuole (Bednarek and Raikhel, 1992). Proteins with vacuolar sorting signals are delivered to the vacuole via transitional vesicles budding from the TGN. Unlike the Man-6-P-dependent sorting of lysosomal hydrolases in mammalian cells, the transport and sorting of vacuolar proteins in plant and yeast cells are not dependent on protein glycosylation, but rather on direct recognition elements within the polypeptide sequence structure (Chrispeels and Raikhel, 1992). Such sorting sequences in yeast vacuolar proteins have been identified in the first 50 N-terminal amino acids of the precursor of carboxypeptidase Y (Valls et al., 1990) and in the first 76 N-terminal amino acids of the precursor of proteinase A (Klionsky et al., 1988).

Some plant proteins contain the vacuolar targeting information either in a CTPP, as BL (Bednarek et al., 1990) and the basic chitinases from tobacco (*Nicotiana tabacum*) (Neuhaus et al., 1991), or in an NTPP, as sporamin (Matsuoka and Nakamura, 1991) and aleurain (Holwerda et al., 1992). Many other vacuolar proteins are synthesized without a cleavable propeptide, indicating that the vacuolar targeting information is contained within the mature protein (Chrispeels and Raikhel, 1992). No amino acid sequence homology is found between C-terminal (Bednarek et al., 1990; Neuhaus et al., 1991) and N-terminal vacuolar sorting signals (Matsuoka and Nakamura, 1991; Holwerda et al., 1992), and it is not known whether the different targeting sequences are recognized by specific sorting determinants.

Abbreviations: BL, barley lectin; CTPP, C-terminal propeptide; GlcNAc, *N*-acetylglucosamine; MS, Murashige and Skoog; NTPP, N-terminal propeptide; TGN, *trans*-Golgi network; WGA, wheat germ agglutinin.

In this study, we compared the ability of BL CTPP and sporamin NTPP to act as vacuolar targeting signals. BL accumulates in small quantities in the outer layers of developing barley (*Hordeum vulgare*) embryos and in root cap cells of barley seedlings (Lerner and Raikhel, 1989). The specific accumulation of lectins such as BL in tissues that establish direct contact with the environment during embryo and seedling development and its insecticidal activity in in vitro experiments suggests that BL is a defense-related protein (Murdock et al., 1990). BL is synthesized as a preproprotein with an N-terminal signal sequence that is cleaved cotranslationally upon translocation into the ER. The proprotein is further modified by glycosylation (high-Man-type glycan) in the CTPP, and during transport or after deposition into the vacuole the glycosylated CTPP is removed from the protein. We have previously demonstrated that BL is processed and targeted correctly in transgenic tobacco (*Nicotiana tabacum*) plants and that the glycan attached to the CTPP is not essential for any of these processes (Wilkins et al., 1990). Recently, we established that the deletion of the CTPP leads to secretion of BL (Bednarek et al., 1990) and that the 15-amino acid CTPP fused to the extracellular protein cucumber chitinase redirects this fusion protein to the vacuole of transgenic tobacco plants (Bednarek and Raikhel, 1991). From these results, we concluded that the CTPP is necessary and sufficient to direct BL to the plant cell vacuole.

Sporamin is a storage protein in tuberous roots of the sweet potato and is synthesized as a preproprotein (Hattori et al., 1985). The N-terminal region of the sporamin precursor contains a signal sequence for translocation across the ER membrane, followed by an NTPP that is 16 amino acids long. The NTPP of the sporamin precursor is essential for the transport of sporamin to the vacuole because a deletion of NTPP from the precursor leads to secretion of sporamin (Matsuoka and Nakamura, 1991). When sporamin is expressed in transgenic tobacco plants, the precursor of sporamin is sequentially processed both cotranslationally and posttranslationally and targeted to the vacuole (Matsuoka et al., 1990).

It is not known whether C-terminal and N-terminal vacuolar targeting signals direct protein transport to the same or different vacuolar compartments. To determine whether the precursors of BL and sporamin are both transported to the same vacuole, we analyzed by immunohistochemistry and pulse-chase labeling experiments the subcellular localization and processing of BL and sporamin in leaf and root tissue of transgenic *N. tabacum* plants expressing both proteins.

MATERIALS AND METHODS

Plant Material

Tobacco plants of *Nicotiana tabacum* cv Wisconsin 38 were transformed with the full-length BL cDNA clone pBLc3 in the pGA643 vector under the transcriptional control of the cauliflower mosaic virus 35S promoter (Wilkins et al., 1990). Plants of *Nicotiana tabacum* cv Bright Yellow No. 4 were transformed with the full-length sporamin cDNA clone pIMO23 in the pMAT037 vector under control of the double cauliflower mosaic virus 35S promoter (Matsuoka and Nak-

amura, 1991). These transgenic plants were cross-pollinated, the seeds from the cross-pollinated plant were germinated, and the F_1 generation selected for plants expressing the BL and sporamin constructs (approximately 25%). Plants were maintained in axenic shoot cultures and propagated by node cuttings on solid MS (Murashige and Skoog, 1962) medium without exogenous hormones.

Isolation and Radiolabeling of Transformed Tobacco Leaf Protoplasts

Protoplasts from transgenic tobacco leaf tissue were prepared as described by Bednarek et al. (1990), with the exception that the cellulase/macerozyme mixture was prepared in MS medium supplemented with 0.1 mg/L of naphthaleneacetic acid, 1.0 mg/L of BA, and 0.6 M betaine monohydrate (MS 0.1/1.0 and 0.6 M betaine). Protoplasts were collected by centrifugation at 50g for 10 min and purified by flotation in MS 0.1/1.0 medium supplemented with 0.6 M Suc, washed once, and diluted to a final concentration of 400,000 protoplasts/mL in MS 0.1/1.0 and 0.6 M betaine. Viable protoplasts were quantified by viability staining with fluorescein diacetate (Widholm, 1972).

For pulse-chase experiments, protoplasts were labeled for 2 h with [^{35}S]Met/Cys and then chased with 1 mM L-Met and 0.5 mM L-Cys as described by Bednarek et al. (1990). At the times indicated, a total of 400,000 protoplasts were harvested by centrifugation and lysed in 200 μL of 50 mM Tris-acetate, pH 5.0, 200 mM NaCl, and 0.5% Triton X-100. To analyze the extracellular protein fraction, the protoplast incubation medium was filtered to remove any remaining protoplasts and NH_4SO_4 was added to a final concentration of 70%. Protoplast protein extracts were prepared by lysis of protoplasts in 10 mM Hepes-KOH, pH 7.5, 0.5% Triton X-100. Insoluble material was removed by centrifugation at 16,000g for 10 min at 4°C.

Vacuole Isolation

Vacuoles were released from the protoplasts by a combination of osmotic and thermal shock as described in detail by Bednarek and Raikhel (1991). Approximately 1 × 10⁷ viable protoplasts 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) preheated to 45°C for 5 min. Released vacuoles were overlaid with 5% (w/v) Ficoll 400 in 0.6 M betaine, 10 mM Hepes-KOH, pH 7.5, and then with 0.6 M betaine, 10 mM Hepes-KOH, pH 7.5, and the gradient centrifuged at 5000g for 30 min at 4°C. The purified vacuoles were recovered from the 0/5% (w/v) Ficoll 400 interface and quantitated using a hemocytometer. Vacuolar protein extracts were prepared as described by Bednarek et al. (1990).

For quantitative immunoblot analysis of the protoplast and vacuolar protein extracts, the activity of the vacuole-specific enzyme α-mannosidase was assayed as described by Boller and Kende (1979). Equal amounts of vacuole and protoplast extracts, relative to the α-mannosidase activity, were precipitated with ice-cold acetone (70% final concentration) for 1 h at −20°C. Samples were resuspended in 30 μL of SDS-PAGE sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 1.0% β-

mercaptoethanol, 10% glycerol, 0.01% bromphenol blue), heated at 95°C for 5 min, and run on a 12.5% SDS-polyacryl-amide gel. Gels were electroblotted onto Immobilon-P (Millipore Corp., Bedford, MA) (Towbin et al., 1979) and the membranes were blocked for 2 h with TBST (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% [v/v] Tween 20) containing 5% nonfat dry milk. The membranes were incubated for 1 h with WGA antiserum diluted 1:1000 in TBSTB (TBST containing 1% BSA). After washing three times for 15 min each in TBSTB, membranes were incubated for 30 min with goat anti-rabbit immunoglobulin conjugated to alkaline phosphatase (Kirkegaard and Perry Labs, Inc., Gaithersburg, MD) diluted 1:5000 in TBSTB. The color reaction visualizing bound secondary antibody was done as described by Blake et al. (1984).

Affinity Chromatography, Immunoprecipitation, and Analysis of Radiolabeled Proteins

All protein samples were passed four times over immobilized GlcNAc (Pierce) microaffinity columns (Mansfield et al., 1988) after extensive washing in TA buffer (50 mM Tris-acetate, pH 5.0, 100 mM NaCl); BL was eluted three times with 50 μL each, 0.2 M GlcNAc, and lyophilized. Sporamin was purified out of the flow-through of the GlcNAc columns by immunoprecipitation. To raise the pH of the extracts to physiological conditions, Tris-Cl (pH 7.5) was added to a final concentration of 100 mM. The extracts were cleared by centrifugation, and 2 μL of sporamin antiserum were added to the supernatant and incubated at room temperature for 1 h. Immunocomplexes were collected on protein A-Sepharose CL-4B beads (Pharmacia, Milwaukee, WI) for 15 min at room temperature and washed three times with TNET250 (25 mM Tris-Cl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% [v/v] Triton X-100). After a final wash 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 on 12.5% SDS-poly-acrylamide gels and visualized by fluorography as described by Mansfield et al. (1988).

Electron-Microscope Immunohistochemistry

Fixation

Small pieces of tobacco leaf tissue were fixed in a mixture of 2% paraformaldehyde and 1% glutaraldehyde in a 50-mM phosphate buffer containing 0.1 M Suc (Bednarek et al., 1990). The tissue was vacuum infiltrated with the fixative and fixed for 2 h at room temperature. Root tips were fixed according to Moore et al. (1991) in 2.5% glutaraldehyde in a 10-mM phosphate buffer. Leaf tissue was postfixed in 1% OsO₄ in 0.01 M sodium phosphate buffer with 0.05 M Suc for 1 h at room temperature. Root tissue was postfixed in 1% OsO₄ in dH₂O. Following dehydration in an ethanol series, leaf and root tissues were embedded in London Resin White acrylic resin (Polysciences, Warrington, PA) and polymerized at 60°C under vacuum. Thin sections were made on an Ultracut microtome (Reichert-Jung, Vienna, Austria) and mounted on formvar-coated nickel grids (Polysciences, Warrington, PA).

Antibody Incubation

Sections were pretreated with 5% Na-meta-periodate (Aldrich Chemical Co., Milwaukee, WI) for 30 min to remove any remaining glutaraldehyde (Craig and Goodchild, 1984) and were blocked two times, for 30 min each, in 2% BSA in PBST (PBS, 0.1% Tween 20). The sections were then treated for 1.5 h with the primary antibody (guinea pig anti-WGA [1:20] or rabbit anti-sporamin [1:100], respectively), diluted in PBST supplemented with 2% BSA. Dilution buffer for anti-WGA antibody was always supplemented with 0.1 M GlcNAc. The sections were washed four times for 5 min each with PBST to remove unbound first antibody and then blocked again two times for 15 min. Bound antibodies were detected with protein A coupled to 10 nm of colloidal gold (EY Laboratories, San Mateo, CA) at a final concentration of 0.05 mg/mL with 1% BSA. For double labeling, thin sections were treated with anti-WGA serum and protein A coupled to 10 nm of colloidal gold, as described. The sections were then saturated for 30 min in PBS supplemented with 1 mg/mL of protein A and incubated with anti-sporamin serum followed by protein A coupled to 15 nm of colloidal gold.

RESULTS

Posttranslational Modifications of BL and Sporamin in Cross-Pollinated Tobacco Plants

To determine whether the precursors of BL and sporamin were targeted to the same plant cell vacuole, we cross-pollinated tobacco plants expressing the precursor of either BL or sporamin. A schematic representation of the full-length cDNA clone pBLc3 (Lerner and Raikhel, 1989) encoding a preproprotein composed of a 2.5-kD signal peptide, an 18-kD polypeptide, and a 1.5-kD CTPP is shown in Figure 1a. The full-length sporamin cDNA clone pIMO23 (Hattori et
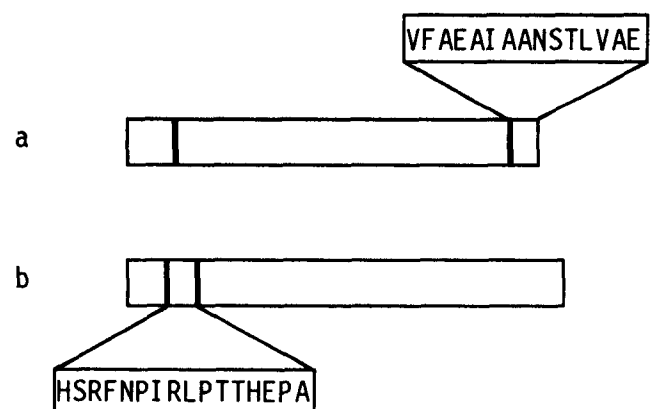
Figure 1. Organization of the BL (a) and sporamin (b) cDNAs. The pBLc3 encodes a polypeptide containing a 26-amino acid signal sequence, followed by the 172-amino acid sequence of the mature protein and the 15-amino acid CTPP (indicated in the box) (Wilkins et al., 1990). The full-length cDNA clone pIMO23 (Hattori et al., 1985) encodes a 21-amino acid signal peptide followed by a 16-amino acid NTPP (indicated in the box) and the 193-amino acid mature sporamin polypeptide.
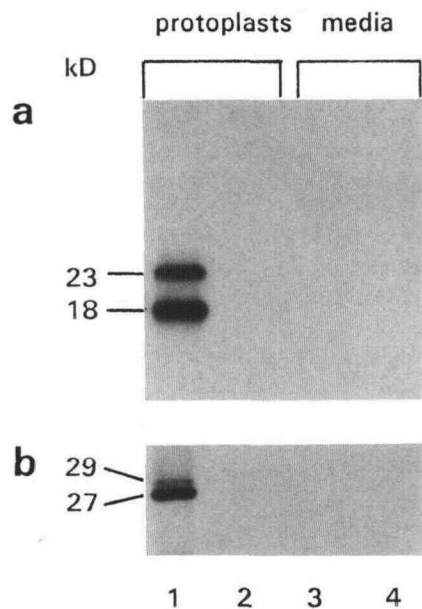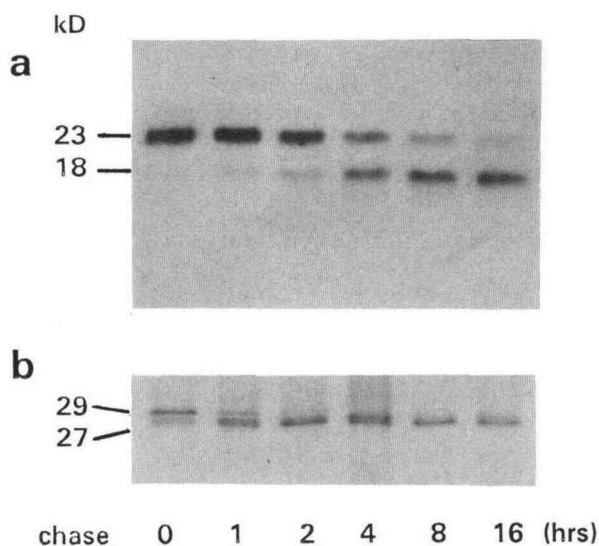
Figure 2. Pulse-labeling experiment of leaf protoplasts isolated from transgenic tobacco (lanes 1 and 3) and untransformed tobacco leaves (lanes 2 and 4) expressing BL and sporamin. Protoplasts were labeled for 16 h with [^{35}S]Met/Cys mix and protein extracts were prepared from protoplasts and the incubation media. BL was purified by affinity chromatography (a) and sporamin was immunoprecipitated (b). The sizes of molecular mass standards are shown on the left in kD.

al., 1985) encodes a 20-kD polypeptide with an N-terminal prepropeptide and contains a typical signal peptide followed by a 1.7-kD NTPP (Fig. 1b). Protein extracts from the cross-pollinated tobacco leaves of the F_1 generation were analyzed by western blot analysis and plants expressing both sporamin and BL were selected. The posttranslational processing of BL and sporamin in the cross-pollinated plants was examined to ascertain whether both proteins were transported correctly to the vacuole or whether missorting of either occurred.

To determine whether any missorting or aberrant processing of BL or sporamin occurred in the cross-pollinated tobacco plants, we labeled leaf protoplasts for 16 h in the presence of a mixture of [^{35}S]Met/Cys. Protein extracts prepared from labeled protoplasts and from the protoplast incubation media were fractionated on an immobilized GlcNAc column (Mansfield et al., 1988). The unbound fraction of the GlcNAc columns was saved and immunoprecipitated with a polyclonal antiserum directed against sporamin. Affinity-purified (Fig. 2a) and immunoprecipitated (Fig. 2b) fractions were analyzed by SDS-PAGE and visualized by fluorography. Two polypeptides corresponding to the 23-kD glycosylated BL proprotein and the mature 18-kD BL were present in pulse-labeled leaf protoplasts (Fig. 2a, lane 1). No radiolabeled proBL or BL was detected in the incubation media recovered from the protoplasts (Fig. 2a, lane 3). As a control, protein fractions from protoplasts of untransformed plants were analyzed, and they did not contain any material bound to the GlcNAc column (Fig. 2, lanes 2 and 4). The protein fraction immunoprecipitated with anti-sporamin serum contained a

predominant polypeptide with an apparent molecular mass of 27 kD corresponding to the mature sporamin, and a minor protein of approximately 29 kD corresponding to the precursor of sporamin (Fig. 2b, lane 1). Neither the precursor nor the mature forms could be detected in the protoplast incubation media (Fig. 2b, lane 3). No cross-reactive material was detected in protoplasts or incubation media of untransformed tobacco plants treated with anti-sporamin serum (Fig. 2b, lanes 2 and 4).

To examine the processing, assembly, and sorting of BL and sporamin, the protoplasts from the cross-pollinated plants were pulse-labeled for 2 h in the presence of [^{35}S]Met/Cys and chased for 16 h in the presence of unlabeled Met/Cys. Protein extracts from the protoplasts were fractionated on a GlcNAc-affinity column and subsequently immunoprecipitated with an anti-sporamin serum (see "Materials and Methods"). As shown in Figure 3a, the 23-kD precursor and the 18-kD mature BL were readily discernible in tobacco leaf protoplasts. After the 2-h pulse, the glycosylated 23-kD intermediate was the most abundant BL form. During the 16-h chase period, most of the precursor was converted into mature BL. The conversion of the precursor was accompanied by a corresponding increase in the amount of radiolabeled mature BL. Based on scanning densitometry, the calculated half-time for the conversion of proBL into mature BL was 2 h. Fluorograms analyzing the immunoprecipitated fraction showed the 29-kD sporamin precursor as the predominantly labeled polypeptide after a 2-h pulse. The 29-kD form was converted with a half-time of 30 min into the mature 27-kD sporamin. The relative amounts of BL and sporamin in protoplasts and vacuolar lysates were compared by immunoblot analysis using anti-WGA serum or anti-sporamin serum. It

Figure 3. Pulse-chase labeling experiment of tobacco leaf protoplasts expressing BL and sporamin. Protoplasts were labeled for 2 h with [^{35}S]Met/Cys mix and chased for 16 h. Protein extracts were prepared from each time point. The extracts were fractionated by affinity chromatography (a) and by immunoprecipitation (b). Proteins were visualized by fluorography. Molecular mass markers are indicated on the left in kD.

was observed that both BL and sporamin were localized quantitatively in the vacuolar fraction (results not shown), confirming that both BL and sporamin were efficiently sorted to the vacuole.
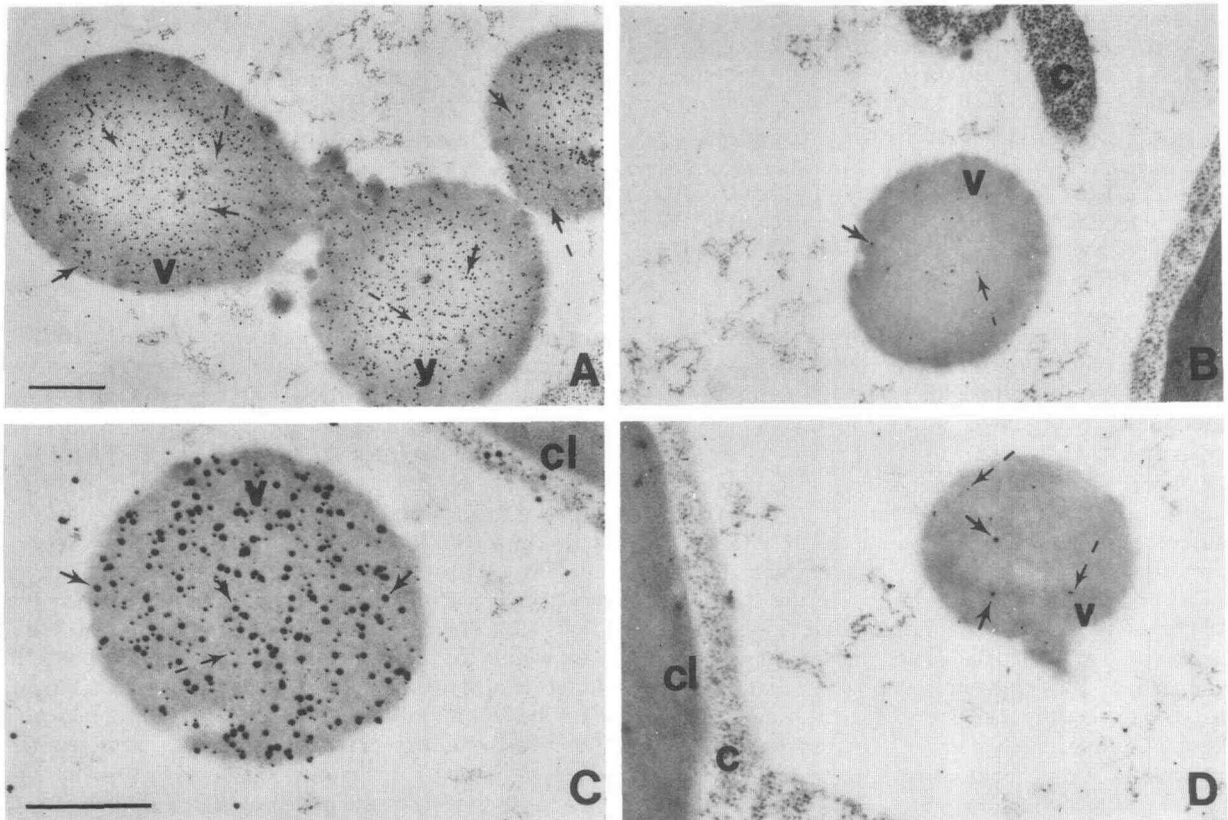
Double-Labeling of BL and Sporamin in Cross-Pollinated Tobacco Plants

To learn whether both BL and sporamin were transported to the same vacuoles in leaf and root plant cells, we examined thin sections by EM and double immunocytochemical analysis. Representative photomicrographs of leaf sections are shown in Figure 4. Electron-dense protein aggregates stained with both anti-WGA (10-nm colloidal gold particles) and anti-sporamin (15-nm colloidal gold particles) were seen in the same vacuole of the transgenic tobacco plants (Fig. 4A). No cross-reactive material was detected in the cell wall or in any subcellular fraction other than the vacuole, which clearly demonstrates that no missorting occurred in the cross-pollinated plants. In no case could we detect vacuolar or vesicular compartments containing either BL or sporamin alone, indicating that both proteins were transported to the same vacuoles. Essentially no labeling was detected in parallel exper-

iments using nonimmune serum instead of the primary antibody (Fig. 4, B and D). Photomicrographs taken at a higher magnification clearly demonstrated the close association of gold particles with the protein aggregates of vacuoles and the even distribution of large and small gold particles within these areas (Fig. 4C). When thin sections of transgenic root tissue were double-labeled with anti-WGA and anti-sporamin sera (Fig. 5, A and C), colloidal gold particles were consistently located in vacuolar protein aggregates similar to those observed in the leaf tissue. No evidence for any missorting or differential vacuolar accumulation could be found. No signal was detected in thin sections treated with nonimmune serum (Fig. 5, B and D). A large number of vacuoles from the leaf and root tissues were analyzed, and in all, both proteins were always observed.

DISCUSSION

The secretory system consists of a series of membrane-bound organelles (ER, Golgi, trans-Golgi, secretory transport vesicles) and delivers secretory proteins to their final intra-or extracellular locations. Sorting of plant vacuolar proteins from other secretory proteins requires specific targeting in-

Figure 4. Double immunogold labeling of BL and sporamin in transgenic tobacco leaf tissue. Thin sections of transgenic tobacco leaves expressing BL and sporamin were treated with anti-WGA serum, and bound antibody was visualized with protein A coupled to 10 nm of colloidal gold. After blocking with an excess of unlabeled protein A, the section was treated with anti-sporamin serum and the bound antibody was visualized with protein A coupled to 15 nm of colloidal gold (A and C). Sections were also treated with the correspondent preimmune serum (B and D). Arrows indicate the 15-nm and 10-nm colloidal gold particle. cl, Chloroplast; c, cytoplasm; v, vacuole. Bars represent 0.5 μm.
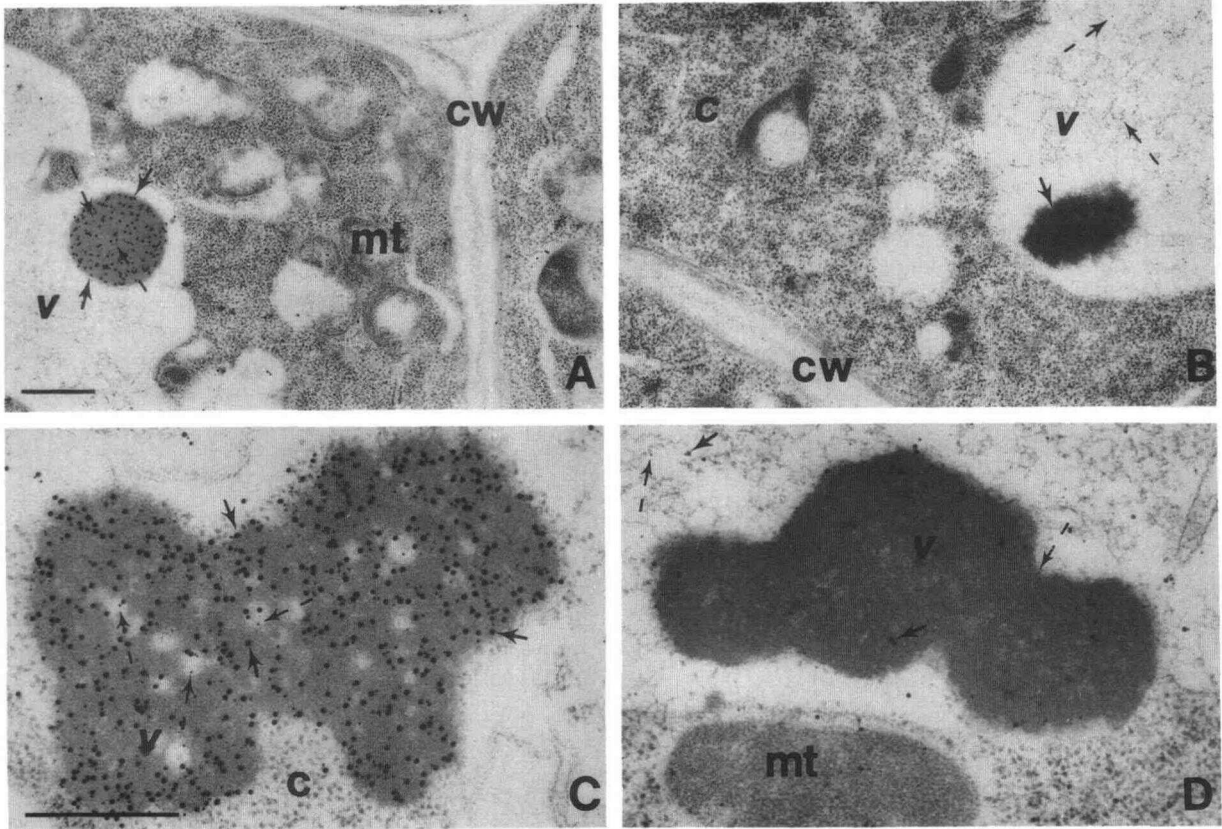
Figure 5. Double immunogold labeling of BL and sporamin in transgenic tobacco root tissue. Thin sections of transgenic root tissue expressing BL and sporamin were labeled with anti-WGA and anti-sporamin serum as described in Figure 4. Specific label in A and C is indicated by arrows. No accumulation of gold particles could be detected in the control section treated with the correspondent preimmune serum (B and D). cw, Cell wall; mt, mitochondria; c, cytoplasm; v, vacuole. Bars represent 0.5 μm.

formation contained within the molecular structures of these polypeptides. In plants, this information is contained within short stretches of amino acids either at the N terminus, the C terminus, or on exposed regions of the mature polypeptide (Chrispeels and Raikhel, 1992).

It was previously demonstrated that signals of either the C terminus (Bednarek et al., 1990; Neuhaus et al., 1991) or the N terminus (Matsuoka and Nakamura, 1991) direct their respective proteins to the vacuole in transgenic tobacco plants. The primary amino acid sequence of the C-terminal vacuolar targeting signals from BL and the tobacco basic chitinases are not conserved. However, the C-terminal extensions are rich in hydrophobic amino acids that may be recognized by the sorting machinery (Bednarek and Raikhel, 1992). Comparison of the sequences of NTPP of the precursors of sporamin and aleuraine reveals a short region (Asn-Pro-Ile-Arg) where Asn and Ile are conserved (Chrispeels and Raikhel, 1992).

Some types of specialized cells (e.g. cells of leaf veins, roots, etc.) contain not one but several vacuoles, and it was not known before this study whether these different vacuolar targeting signals direct protein transport to the same or different vacuolar compartments. Our results using double-labeling immunohistochemistry of thin sections obtained

from transgenic tobacco plants expressing BL (CTPP) and sporamin (NTPP) indicate that both proteins are delivered to the same vacuoles in transgenic tobacco plants. Although there is no sequence homology between C-terminal and N-terminal targeting signals (Bednarek and Raikhel, 1992), the results of the double-immunolabeling experiments suggest that both proteins are equally recognized by the vacuolar protein-sorting machinery.

However, we cannot exclude the possibility that there are certain specialized cells that indeed have different types of vacuoles, and it remains to be shown if there are signal sequences that could mediate differential transport. There is no evidence to date that other secretory proteins are targeted by the sorting machinery to different types of vacuoles, or bypass the Golgi. However, prolamins, representing seed storage proteins in the developing endosperm of rice and maize, aggregate in the lumen of the ER (Larkins and Hurkman, 1978; Krishnan et al., 1986). These aggregated prolamins are sequestered in ER-derived compartments, so-called protein bodies, that are clearly distinguished from protein storage vacuoles. Neither the sorting signal that causes accumulation of protein in the ER nor the exact mechanism of protein body formation is known (for review, see Bednarek and Raikhel, 1992).

Pulse-chase labeling experiments of protoplasts from the cross-pollinated tobacco plants showed that the posttranslational processing of the sporamin precursor into the mature protein occurs four to five times faster than the processing of proBL, mimicking behavior of both propeptides in their respective parent plants (Bednarek et al., 1990; Matsuoka and Nakamura, 1991). Because BL is a dimer with 16 disulfide bonds per subunit, and sporamin is a monomer, it is possible that the difference in processing efficiency may be due to the time required to form the correct oligomeric structure of BL compared with the monomeric sporamin. Recently, it has been shown that the trimerization of the pea vacuolar protein phaseolin is required for efficient sorting (Ceriotti et al., 1991). In addition, it is known that in mammalian cells oligomerization and assembly are required for the transport of immunoglobulin from the ER (Hurtley and Helenius, 1989). However, the different speed of posttranslational processing of proBL and prosporamin may reflect a real difference in the sorting efficiency of CTPP and NTPP. This is supported by redirection experiments using extracellular cucumber chitinase as a reporter protein fused to CTPP, and suggests that these targeting elements have to be presented in the proper secondary and/or tertiary structural context to be recognized efficiently (Bednarek and Raikhel, 1991).

Because there is no consensus sequence between the CTPP and the NTPP, the individual amino acid composition may influence the efficiency of recognition by the sorting machinery and, therefore, the time needed to reach the compartment where the propeptide is cleaved. Because there was no indication of missorting of either BL or sporamin in the cross-pollinated tobacco plants, we hypothesize that both peptides are recognized by either the same abundant, broad-specificity sorting mechanism or by two different ones.

CONCLUSIONS

We have previously shown that the CTPP of BL and the NTPP of sporamin are necessary to direct these proteins to the plant vacuole. Although the BL CTPP and the sporamin NTPP share no amino acid sequence homology, both proteins were localized in the same vacuoles when expressed simultaneously in transgenic tobacco plants. No indication of any competition between proteins was observed in plants expressing BL and sporamin.

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