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Different Sensitivity to Wortmannin of Two Vacuolar Sorting Signals Indicates the Presence of Distinct Sorting Machineries in Tobacco Cells

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Abstract. Vacuolar matrix proteins in plant cells are sorted from the secretory pathway to the vacuoles at the Golgi apparatus. Previously, we reported that the NH_2-terminal propeptide (NTPP) of the sporamin precursor and the COOH-terminal propeptide (CTPP) of the barley lectin precursor contain information for vacuolar sorting. To analyze whether these propeptides are interchangeable, we expressed constructs consisting of wild-type or mutated NTPP with the mature part of barley lectin and sporamin with CTPP and mutated NTPP in tobacco BY-2 cells. The vacuolar localization of these constructs indicated that the signals were interchangeable. We next analyzed the effect of wortmannin, a specific inhibitor of mammalian phosphatidylinositol (PI) 3-kinase on vacuolar delivery by NTPP and CTPP in tobacco cells. Pulse–chase analysis indicated that 33 μM wortmannin caused almost complete inhibition of CTPP-mediated transport to the vacuoles, while NTPP-mediated transport displayed almost no sensitivity to wortmannin at this concentration. This indicates that there are at least two different mechanisms for vacuolar sorting in tobacco cells, and the CTPP-mediated pathway is sensitive to wortmannin. We compared the dose dependencies of wortmannin on the inhibition of CTPP-mediated vacuolar delivery of proteins and on the inhibition of the synthesis of phospholipids in tobacco cells. Wortmannin inhibited PI 3- and PI 4-kinase activities and phospholipid synthesis. Missorting caused by wortmannin displays a dose dependency that is similar to the dose dependency for the inhibition of synthesis of PI 4-phosphate and major phospholipids. This is different, however, than the inhibition of synthesis of PI 3-phosphate. Thus, the synthesis of phospholipids could be involved in CTPP-mediated vacuolar transport.

In many plant cells the vacuole is the largest organelle, and it has diverse functions. It is a lytic compartment for the intracellular digestion of materials (equivalent to mammalian lysosomes), it is involved in the storage of organic and inorganic nutrients and metabolites, and is important for the generation of turgor and cell growth, with specific function depending on the type and stage of development of the cell (Boller and Wiemken, 1986).

Most soluble proteins destined for the vacuole in plants and yeast or the lysosome in mammalian cells are synthesized by membrane-bound polysomes as precursors with NH_2-terminal signal peptides. After synthesis and translocation across the ER membrane, the signal peptides are removed and, upon folding, the proteins are transported to the Golgi apparatus, where they are sorted at the *trans*-Golgi network to the vacuole/lysosome. Sorting to the vacuole or lysosome, as well as retention within organelles of the secretory pathway, requires specific targeting information. Soluble proteins lacking this information are transported through the secretory pathway and are secreted to the extracellular space (Bednarek and Raikhel, 1992). The targeting signals have now been well characterized in a number of proteins and have been found to differ between mammalian, yeast, and plant cells. The signal for sorting to mammalian lysosomes is the phosphorylation of mannose residues, which enables the proteins to be recognized by mannose-6-phosphate receptors and transported to the lysosome (Kornfeld and Mellman, 1989). In yeast cells, proteins destined for the vacuole are synthesized with an NH_2-terminal propeptide that functions as a targeting signal and is removed upon deposition in the vacuole (Rothman et al., 1989). In plant cells the situation appears more complex. Three types of targeting signal have been found for the sorting of proteins to the vacuole. Some proteins contain an NH_2-terminal propeptide (NTPP)[1] as a target-

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1. *Abbreviations used in this paper*: BL, barley lectin; CTPP, COOH-terminal propeptide; NTPP, NH_2-terminal propeptide; P100, pelleted membranous organelles; PI, phosphatidylinositol; S1, supernatant total cell homogenate; S100, supernatant soluble fraction; SPO, sporamin.

Published September 15, 1995

ing signal, some contain a COOH-terminal propeptide (CTPP), and others contain a targeting signal within the mature region of the protein (Chrispeels and Raikhel, 1992; Nakamura and Matsuoka, 1993). However, these plant vacuolar targeting signals are not recognized in yeast, suggesting that at least some components of the transport machinery are different between yeast and plants (Gal and Raikhel, 1993).

We have been analyzing the mechanisms for the transport of proteins to the vacuole in plant cells using sweet potato sporamin and barley lectin (BL) as model proteins. Sporamin is a vacuolar protein of the tuberous roots of the sweet potato and comprises ~80% of the soluble proteins in the tuberous roots (Maeshima et al., 1985). It is a monomeric, water-soluble protein of ~20 kD. Its precursor is composed of a signal peptide, a 16–amino acid NTPP containing the vacuolar targeting information (Matsuoka and Nakamura, 1991) and the mature part of the protein. When the sporamin precursor was expressed in tobacco cells, the proprotein was O-glycosylated in the Golgi apparatus, although O-glycosylation was not necessary for transport to the vacuole (Matsuoka et al., 1995). The amino acid sequence Asn-Pro-Ile-Arg in the NTPP is important for the vacuolar sorting of the sporamin precursor since mutations in this region cause a decrease in the sorting efficiency (Nakamura et al., 1993).

BL is a homodimeric lectin that binds to *N*-acetylglucosamine (for review see Raikhel and Lerner, 1991). BL is synthesized on the ER as a precursor with a signal sequence and a 15–amino acid CTPP, which is N-glycosylated with high-mannose–type glycan. The N-linked glycan is not necessary for the sorting of this protein to the vacuole, although it modulates the rate of processing of the propeptide (Wilkins et al., 1990). The CTPP is both necessary and sufficient for the targeting of BL to the vacuole (Bednarek et al., 1990; Bednarek and Raikhel, 1991). Mutagenesis of the CTPP demonstrated that a wide variety of amino acid sequences can be used as a vacuolar targeting signal (Dombrowski et al., 1993).

Although sporamin and BL are transported to the same vacuoles when they are coexpressed in tobacco plants (Schroeder et al., 1993), there are no similarities between the sequences of the NTPP and the CTPP. A putative vacuolar targeting receptor protein has recently been identified that is able to bind to an affinity column containing the proaleurain NTPP. A peptide consisting of the prosporamin NTPP is able to compete for binding, whereas the pro-BL CTPP is not (Kirsch et al., 1994). This suggests that different receptor proteins, and possibly targeting mechanisms, are involved in the transport of proteins containing NTPP and CTPP. To examine whether NTPP- and CTPP-mediated vacuolar delivery involves different mechanisms in tobacco cells, we first analyzed whether the propeptides of prosporamin and pro-BL are interchangeable with each other. The CTPP and NTPP were found to be functionally interchangeable in their ability to direct each of these proteins to the vacuole. We then went on to investigate the mechanisms by which these proteins are delivered to the vacuole. In yeast, the *VPS34* gene is essential for vacuolar protein sorting and encodes a protein with phosphatidylinositol (PI) 3-kinase and protein kinase activities (Stack et al., 1993; Stack and Emr, 1994). PI 3-kinases were recently cloned from *Arabidopsis thaliana* and soybean, which show homology to VPS34p (Welters et al., 1994; Hong and Verma, 1994), although the *Arabidopsis* gene is not able to complement the yeast *vps34* mutant (Welters et al., 1994). We therefore analyzed the effect of the reagent wortmannin, an inhibitor of PI kinase (Thelen et al., 1994), on vacuolar delivery of NTPP- and CTPP-containing proteins, and on the synthesis of phospholipids in tobacco cells. The results obtained suggest that at least two different mechanisms exist for the transport of soluble proteins to the vacuole in plants.

Materials and Methods

Wortmannin (Sigma Chemical Co., St. Louis, MO), was dissolved to 10 mM in DMSO and stored at −20°C. Antibodies against SDS-denatured sporamin and native sporamin were as described (Matsuoka et al., 1990). Antibody against denatured wheat germ agglutinin (WGA) were as described in Dombrowski et al. (1993). Antibody against tobacco class I chitinase was a kind gift of Dr. W. F. Broekaert (Catholic University of Leuven, Heverlee, Belgium), and antibody against tobacco endo β-1,3-glucanase was a kind gift of Dr. F. Meins, Jr (Friedrich Miescher Institute, Basel, Switzerland).

Construction of Mutants and Transformation into BY-2 Tobacco Cells

BL fusion constructs were synthesized by recombinant PCR. The BL coding region of pTaw50 (Bednarek et al., 1990) was amplified using CACCACACACCAGGTGCGGCGAGCAGGGCA and universal M13 primers. The region encoding the signal peptide and propeptide of sporamin was amplified from pMAT103 (wild type) and pMAT178 (I28G mutant) using CGCACCTCTGGTGTGTGGTGGGGAGGCGG and SP6 primers (Matsuoka and Nakamura, 1991; Nakamura et al., 1993). These first-round PCR products were mixed and reamplified with M13 and SP6 primers, and the resulting fragment was subcloned into the pNUT7 plasmid (Matsuoka and Nakamura, 1991). The DNA segment encoding the COOH-terminal half of proBL was exchanged with the CTPP-minus BL segment from pTaw53 (Bednarek et al., 1990), when required.

For sporamin-based constructs, the BL CTPP was amplified by PCR using TGCTCTAGAGTCTTCGCCGAGGCCATCGCC and commercial M13 primers and subcloned into a derivative of pMAT103 (Matsuoka and Nakamura, 1991) at an XbaI site introduced immediately after the sporamin coding region. The region of DNA encoding the NH_2-terminal portion of sporamin was exchanged with the Δpro, N26G, and I28G mutants of prosporamin when required.

All of the DNA sequences of the mutant constructs were confirmed by dideoxy nucleotide sequencing (Sanger et al., 1977). Mutant cDNAs were placed downstream of the cauliflower mosaic virus 35S promoter in the binary vector pMAT037 and used for *Agrobacterium*-mediated transformation of BY-2 suspension-cultured tobacco cells (Matsuoka and Nakamura, 1991).

Culture and Fractionation of Tobacco Cell Culture and Detection of Expressed Proteins

Transformed tobacco cell colonies (300–3,000) were pooled and reintroduced to suspension culture. These cultures were subcultured weekly (Matsuoka and Nakamura, 1991) and analyzed within 1 yr of transformation. Preparation of medium, cells, protoplasts, and vacuoles from mid–log phase culture of the transformed tobacco cells was as described (Matsuoka and Nakamura, 1991). Sporamin- or BL-related proteins in each fraction were detected by immunoblotting after 12.5% SDS-PAGE (Matsuoka et al., 1990; Lerner and Raikhel, 1989). BL- or sporamin-related proteins were recovered from cell lysates using an *N*-acetylglucosamine affinity column (Pierce; Wilkins et al., 1990) or immunoaffinity column (Matsuoka et al., 1990), respectively, and the NH_2-terminal sequences analyzed by Edman degradation essentially as described (Matsuoka et al., 1990).

Analysis of Transformants by Pulse–Chase Labeling

Analysis of the secretion and processing of proteins in the transformed cells by pulse–chase labeling with ^{35}S-labeled amino acids was performed

Published September 15, 1995

essentially as described previously (Matsuoka et al., 1990; Matsuoka and Nakamura, 1991). In brief, 2.8 MBq of Tran^{35}S-label (ICN Biomedicals, Inc., Costa Mesa, CA) was shaken with 500 μl of a suspension of tobacco cells for 15 min at 28°C. 50 μl of a solution of 50 mM Met and 10 mM Cys was added with further shaking for appropriate chase periods. If required, wortmannin was added to the cells at the appropriate concentration for 30 min before pulse labeling. Labeled sporamin, BL, class I chitinase, or β-1,3-glucanase was recovered by immunoprecipitation (Matsuoka et al., 1990). The immunoprecipitated proteins were separated by SDS-PAGE and detected by autoradiography or by phosphorimaging with an image analyzer (BAS 2000; Fuji Photo Film, Tokyo, Japan). The quantitative analysis of each band was done by the image analyzer.

Extraction of Proteins for the Analysis of PI 3-Kinase Activity

A yeast cell lysate was prepared from logarithmically grown cells of a protease-deficient diploid yeast strain, as described by Schu et al. (1993). Rat spleen lysate was prepared from a 220-g male wister rat as described by Otsu et al. (1991), except that 50 mM Hepes-KOH buffer was used instead of Tris buffer.

For the preparation of a tobacco cell lysate, tobacco cells of mid–log phase were collected by centrifugation, after treatment with wortmannin if required, washed twice with homogenization buffer (15 mM Hepes-KOH, pH 7.5, 10% glycerol, 0.1 M KCl, 3 mM EGTA, 1 mM DTT, 1 mM PMSF) and suspended in an equal volume of the homogenization buffer. The cell suspension was homogenized using a Potter-Elvehjeom homogenizer and centrifuged at 1,000 *g* for 10 min. The supernatant (total cell homogenate; S1) was stored at −80°C until use. For the separation of particulate and soluble fractions, the total cell homogenate was centrifuged at 100,000 *g* for 1 h. The supernatant, referred to as the soluble fraction (S100), was stored at −80°C. The pelleted membranous organelles (P100) were resuspended to the original volume in homogenization buffer and stored at −80°C.

PI-Kinase Assay

PI 3-kinase activity was measured essentially as described by Schu et al. (1993), except that γ-[^{33}P]ATP (Amersham Co., Tokyo, Japan) was used instead of γ-[^{32}P]ATP. Briefly, the cell lysates were preincubated with PI and reaction buffer for 3 min at 25°C to equilibrate the reaction mixture to reaction temperature. The kinase reaction was started by addition of radiolabeled ATP and incubated for 5 min at 25°C. The reaction was stopped by the addition of chloroform and methanol (1:1). The extracted phospholipids were dried under vacuum, dissolved in chloroform, and analyzed by TLC on a silica gel 60 plate (Whatmann Inc., Clifton, NJ) impregnated with *trans*-1,2-diaminocyclohexane-N,N,N′,N′-tetraacetic acid as described (Walsh et al., 1991). The plate was developed using a borate solvent system (Walsh et al., 1991) and phospholipids were detected and quantified using a BAS 2000 image analyzer. To analyze the sensitivity of PI 3-kinase activity to wortmannin, wortmannin solution was added to the sample 1 min before the start of the reaction. In some cases, phospholipids were extracted from the silica gel as described (Walsh et al., 1991), further analyzed on a potassium oxalate–impregnated silica gel 60 TLC plate with concentration zone (Whatmann Inc.), and developed with an alkaline solvent (Munnik et al., 1994).

Metabolic Labeling of Phospholipids in Tobacco Cells by [^{32}P]Phosphate and Analysis of the Labeled Phospholipids

Wortmannin solution was added at an appropriate concentration to 500 μl of a tobacco cell suspension at mid–log phase and incubated for 45 min at 28°C with shaking. 1.85 MBq of carrier-free [^{32}P]phosphate (Amersham) was added and the incubation continued for a further 90 min. ^{32}P-labeled phospholipids were extracted essentially as described (Munnik et al., 1994). Briefly, labeled cells were mixed with 600 μl $CHCl_3/CH_3OH/HCl$ (100:100:1 by volume), and the lipids were directly extracted while rapidly freezing the cells in liquid nitrogen and thawing. Subsequently, cells were pelleted by centrifugation (14,000 *g* for 2 min) and the supernatants collected. Cells were reextracted with $CHCl_3/CH_3OH/HCl$ (100:100:1 by volume) as above. Lipid fractions were pooled, 500 μl $CHCl_3$ and 375 μl 0.9% NaCl added, and the two-phase system was centrifuged for 2 min as above. The organic lower phase was washed three times with $H_2O/CHCl_3/HCl$ (50:50:1 by volume) and separated using an alkaline or borate TLC system. The radioactivity of labeled phospholipids were quantified by use of a BAS 2000 image analyzer. For the identification of each spot on the alkaline TLC plates, the Rf of each spot was compared with authentic samples of each phospholipid (Sigma Chemical Co.). Alternatively, radiolabeled phospholipids were extracted from the silica matrix as described above, separated using an acidic TLC system (Munnik et al., 1994), and the Rf value of the spots were compared with authentic samples. The authentic samples of phospholipids on alkaline or acidic TLC plates were detected by iodine vapor as described (Munnik et al., 1994).

Results

The Prosporamin NTPP and the pro-BL CTPP Are Interchangeable for the Targeting of Proteins to the Vacuole

We reported previously that the prosporamin NTPP and the pro-BL CTPP contain information for vacuolar sorting in tobacco cells (Matsuoka and Nakamura, 1991; Bednarek and Raikhel, 1991). In this study, we first analyzed whether these propeptides are interchangeable with the mature proteins. To test whether the NTPP can direct BL to the vacuole, we made constructs consisting of NTPP-BL (NTPP fused to mature BL), I28G-BL (mutant NTPP fused to mature BL), and I28G-BL-CTPP (mutant NTPP fused to pro-BL) (Fig. 1). The I28G mutation (Ile^{28} to Gly) in the NTPP renders it nonfunctional and thus causes almost complete secretion of sporamin to the medium (Nakamura et al., 1993; Fig. 2, *c* and *d*; see Fig. 4, I28G-SPO).

These constructs, and BL with and without the CTPP, were placed under control of the 35S promoter of the cauliflower mosaic virus, and the proteins were expressed in tobacco BY-2 suspension-cultured cells. The distribution of BL-related proteins between the medium and cells was analyzed by Western blotting using anti-WGA antibody (which also recognizes BL; Lerner and Raikhel, 1989; Fig. 2 *a*). The culture medium of the transformed cells expressing either BL without CTPP or I28G-BL contained BL-related polypeptides (Fig. 2 *a*), while the media from cells transformed with NTPP-BL, I28G-BL-CTPP, or BL-CTPP did not contain detectable amounts of BL, which was exclusively found intracellularly. This indicates that the wild-

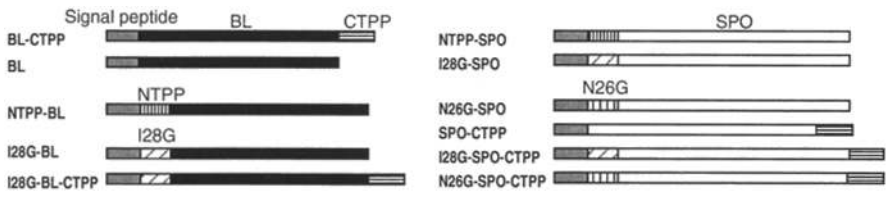

Figure 1. Schematic representation of constructs used in this study. The terms used for each construct are shown on the left. Regions of constructs are distinguished graphically. CTPP of pro-BL is both necessary and sufficient for the transport of BL to the vacuole (Bednarek et al., 1990; Bednarek and Raikhel, 1991); NTPP of prosporamin contains vacuolar targeting information (Matsuoka and Nakamura, 1991); I28G is a completely nonfunctional mutant of NTPP in vacuolar targeting with Ile^{28}→Gly substitution (Nakamura et al., 1993); N26G is a partially functional mutant of NTPP in vacuolar targeting with Asn^{26}→Gly mutation (Nakamura et al., 1993).

Published September 15, 1995

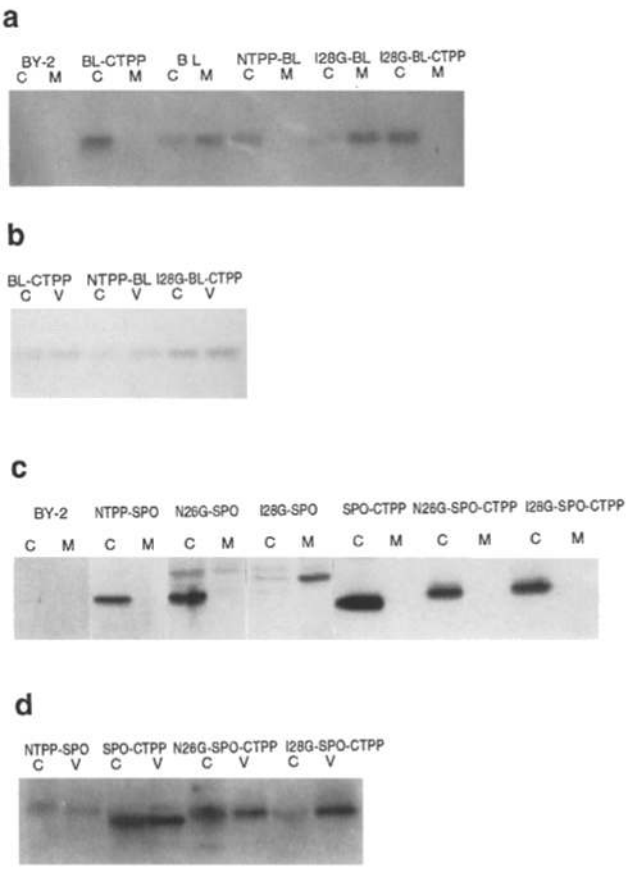

Figure 2. Distribution of SPO and BL fusion proteins in the tobacco cell culture. (*a*) Distribution of BL in the cells and medium of transformed tobacco cells expressing BL constructs. Proteins from the cells and medium of mid–log phase tobacco cell cultures were separated by SDS-PAGE, transferred to polyvinilidene fluoride (PVDF) membrane, and BL-related proteins were detected by immunoblotting using anti-WGA antibody. BY-2 indicates nontransformed BY-2 cells. (*b*) Intracellular localization of BL-CTPP, NTPP-BL, and I28G-BL-CTPP in transformed tobacco cells. Vacuoles were prepared from protoplasts of transformed tobacco cells. Proteins from cells and vacuoles, containing equivalent α-mannosidase activities, were separated by SDS-PAGE, and BL was detected as in *a*. (*c*) Distribution of SPO in the cells and medium of transformed tobacco cells expressing SPO constructs. SPO was detected as in *a* using anti-SPO antibody. (*d*) Intracellular localization of NTPP-SPO, SPO-CTPP, N26G-SPO-CTPP, and I28G-SPO-CTPP in transformed tobacco cells. SPO in cells or vacuoles was detected as in *a* using anti-SPO antibody. *C*, cells; *M*, medium; *V*, vacuoles.

type NTPP caused the accumulation of BL in the cell and confirmed our previous result (Nakamura et al., 1993) that the Ile^{28}-to-Gly substitution mutation causes the disruption of vacuolar targeting. To determine whether the NTPP of NTPP-BL is processed, we prepared BL-related polypeptides by *N*-acetylglucosamine affinity chromatography from transformed tobacco cells expressing NTPP-BL and analyzed the NH_2-terminal sequence by Edman degradation. The sequence Thr-Thr-X-Gln-Arg-Gly-Glu-Gln-X-X-Asn indicates that the NH_2 terminus of the polypeptide starts at the 32nd amino acid (Thr) of the precursor. Since the length of the signal peptide of the prosporamin precursor is ~23 amino acids, this result suggests that the propeptide of NTPP-BL is posttranslationally processed in the cells.

To confirm that NTPP directs BL to the vacuoles in tobacco cells, vacuolar fractions were analyzed by Western blotting (Fig. 2 *b*). The almost identical intensity of the band corresponding to BL in the protoplasts and the vacuoles, relative to the activity of α-mannosidase, a vacuolar marker enzyme, indicated that these BL-related proteins in the cells are transported to the vacuole. This confirms that the sporamin propeptide is both necessary and sufficient for the transport of proteins to the vacuole.

To determine whether the CTPP can direct sporamin (SPO) to the vacuole, we synthesized SPO-CTPP, consisting of mature SPO with the BL CTPP, I28G-SPO-CTPP, and N26G-SPO-CTPP, and analyzed the fate of these proteins in tobacco cells. The I28G-mutated NTPP is completely nonfunctional in vacuolar targeting, whereas the N26G NTPP is partially functional, being able to direct ~60% of SPO to the vacuole (Nakamura et al., 1993). In contrast to the case of another reporter protein, cucumber chitinase–CTPP (Bednarek and Raikhel, 1991), no significant amount of SPO was observed in the culture media of the transformed tobacco cells expressing any of these constructs (Fig. 2 *c*). The migration on SDS-PAGE of the intracellular SPO-related polypeptides suggested that the CTPP was proteolytically removed. The NH_2-terminal amino acid sequence of SPO from transformed tobacco cells that expressed the I28G-SPO-CTPP construct was determined after purification of SPO from the transformed cells by immunoaffinity chromatography. The amino acid sequence was determined to be Glu-Pro-Ala-Ser-X-Glu-Thr-Pro-Val, identical to that of NTPP-SPO expressed in tobacco cells (Matsuoka et al., 1990). This indicates that the site of the processing of the NTPP is independent of its vacuolar targeting function. To address whether these proteins were correctly targeted to the vacuole, we prepared vacuoles from the transformed tobacco cells, and the relative amount of SPO compared with the α-mannosidase activity was determined by Western blotting (Fig. 2 *d*). This indicates that these SPO-related proteins in the cells accumulated in the vacuoles. These results demonstrate that the prosporamin NTPP and pro-BL CTPP are functionally interchangeable.

The Rate of Transport of Proteins to the Vacuole Is Determined Mainly by the Protein Being Transported, Not by the Targeting Signal

We have previously shown that the rate of transport of BL-CTPP and NTPP-SPO to the vacuole differs when the proteins are expressed in the same tobacco plant (Schroeder et al., 1993). To address whether this difference is due to the different targeting signals or to characteristics of the mature proteins such as the rate of folding or posttranslational modification, we analyzed the kinetics of transport of various constructs by pulse–chase analysis. Tobacco cells expressing each of the constructs were pulse labeled for 15 min with ^{35}S-amino acids and chased with excess unlabeled Met and Cys for various times. BL- or sporamin-related proteins in the cell and medium fractions of the culture were recovered by immunoprecipitation, separated by SDS-PAGE, and ^{35}S-labeled proteins in the gels

Published September 15, 1995

were detected by autoradiography or phosphorimage analysis.

In the case of the BL-CTPP transformants, pulse-labeled cells gave a 24-kD band that corresponds to the native BL-CTPP protein with an N-linked glycan on the CTPP (Wilkins et al., 1990; Fig. 3, *BL-CTPP, cells, 0 min*). After a 60-min chase, about half of this precursor form was converted into the vacuolar form of BL migrating at 18 kD, and after a 240-min chase, most of the proform was converted to the mature form (Fig. 3, *BL-CTPP*). No secretion of BL was observed during the chase periods. The kinetics of vacuolar transport of I28G-BL-CTPP were similar to those of BL-CTPP. Pulse labeling of the NTPP-BL transformant gave an 18.5-kD band corresponding to BL containing the NTPP (Fig. 3, *NTPP-BL*). By 240 min of the chase, most of the proform was converted to the vacuolar form of 18 kD (Fig. 3, *NTPP-BL*). No secretion of BL-related proteins was observed during the chase periods (Fig. 3, *NTPP-BL*). The half-times for the formation of the 18-kD vacuolar forms were calculated as ~40–50 min in each case. In the case of the BL or I28G-BL transformants, pulse-labeled BL-related polypeptides were secreted to the medium (Fig. 3, *BL* and *I28G-BL*).

NTPP-SPO precursor (26.8 kD) was converted to the mature form of 24.0 kD with a half-time of ~21 min (Fig. 4, *NTPP-SPO*). The smeared bands represent O-glycosylated forms (Matsuoka et al., 1995). SPO-CTPP, however, was converted to the vacuolar form very slowly, although very little was secreted to the medium, and thus it appears that NTPP is required for transport competence of this protein, and possibly for its correct folding in the ER (data not shown). In the case of the I28G-SPO-CTPP and N26G-SPO-CTPP transformants, similar kinetics of processing were observed as for NTPP-SPO (Fig. 4, *I28G-SPO-CTPP*), demonstrating that an NTPP that is nonfunctional or partially functional for vacuolar targeting can still promote the folding or transport competence of SPO. Pulse-labeled I28G-SPO was secreted to the medium with similar kinetics as for SPO without a propeptide (Fig. 4, *I28G-SPO*; Matsuoka and Nakamura, 1991). The secreted I28G-SPO was proteolytically processed in the medium between Gly^{28} and Arg^{29} (data not shown). These observations suggest that the rate of transport to the vacuole is determined mainly by properties of the protein being transported, such as its rate of folding, and not by the targeting signal.

Effect of Wortmannin on the Sorting of the Sporamin and BL Constructs

It has been demonstrated that PI 3-kinase activity is involved in vesicle transport and membrane structure in various cell types. The sorting of proteins to the vacuole in yeast cells requires PI 3-kinase activity (Schu et al., 1993), and recent analysis suggests that a PI 3-kinase in soybean cells is associated with membrane proliferation during nodule development (Hong and Verma, 1994). The PI 3-kinase in soybean cells resembles both the mammalian and yeast

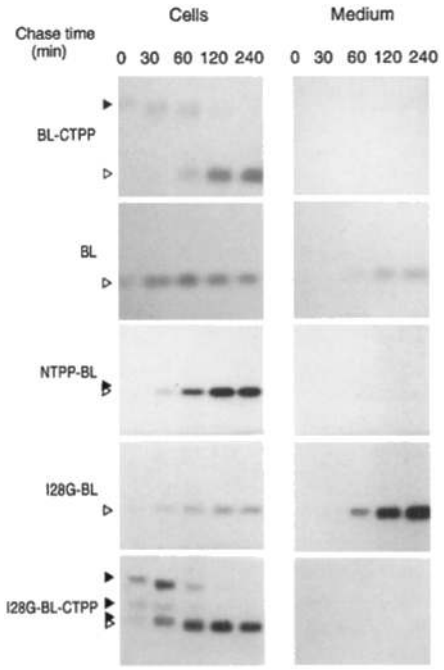

Figure 3. Pulse–chase analysis of BL in transformed tobacco cells expressing BL constructs. Cultures were pulse labeled with ^{35}S-amino acids for 15 min and chased with unlabeled methionine and cysteine for the indicated periods of time. BL-related polypeptides in the cells and the medium from equal volumes of culture were immunoprecipitated with anti-WGA antibodies, separated by SDS-PAGE, and detected by autoradiography. The solid and open arrowheads indicate the migration position of the precursor and mature forms of the BL constructs, respectively.

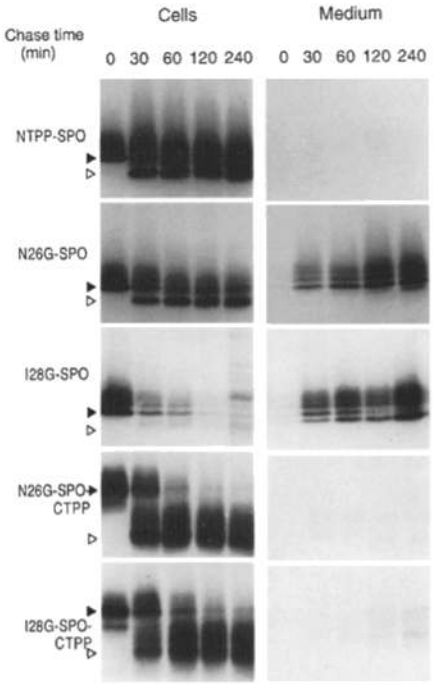

Figure 4. Pulse–chase analysis of SPO in transformed tobacco cells expressing SPO constructs. Cultures pulse labeled with ^{35}S-amino acids for 15 min were chased with unlabeled methionine and cysteine for the indicated periods of time. SPO-related polypeptides in the cells and medium from equal volumes of culture were immunoprecipitated with SPO-specific antibodies, separated by SDS-PAGE, and detected by autoradiography. The solid and open arrowheads indicate the migration position of the precursor and mature forms of the SPO constructs, respectively.

Published September 15, 1995

PI 3-kinases. Recently it was reported that a fungal metabolite, wortmannin, is a potent and specific inhibitor of mammalian PI 3-kinase (Yano et al., 1993; Arcaro and Wymann, 1993) and thus is expected to disrupt membrane transport and structure.

To address the question of whether wortmannin inhibits the sorting of proteins to the vacuole by NTPP and by CTPP in tobacco cells, we analyzed the transport of the BL and SPO constructs in the presence of various concentrations of wortmannin by pulse–chase analysis (Figs. 5 and 6). Up to 33 μM wortmannin had no effect on the incorporation of ^{35}S-amino acids into the proteins during the 15-min pulse periods, while 100 μM wortmannin caused a decrease in the incorporation of ^{35}S-amino acids into proteins by ~70% (data not shown). In the presence of 33 μM wortmannin, almost all of the pulse-labeled BL-CTPP or I28G-BL-CTPP were secreted to the medium during the

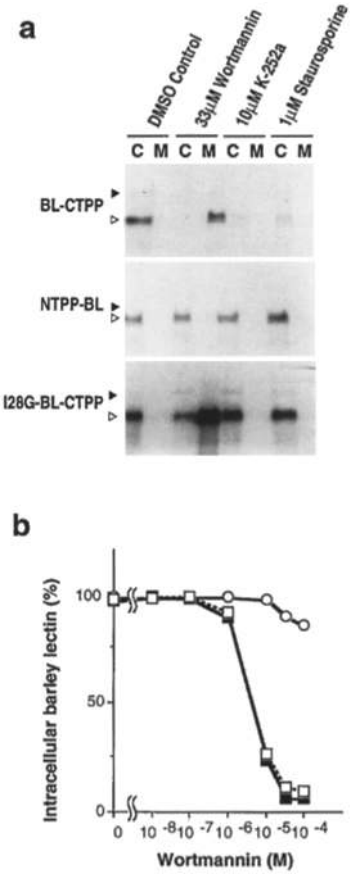

Figure 5. Effects of wortmannin on the transport of wild-type and mutant BL. (*a*) Transformed tobacco cells preincubated with either wortmannin (33 μM), K-252a (10 μM), or staurosporine (1 μM) for 30 min were pulse labeled for 15 min and chased for 240 min in the presence of inhibitors. BL-related proteins in cells or medium were analyzed as in Fig. 3. The solid and open arrowheads indicate the migration position of the precursor and mature forms of the BL constructs. *C*, cells; *M*, medium. (*b*) Dose-dependent secretion of BL constructs in the presence of wortmannin. Transformed tobacco cells were pulse labeled and chased in the presence of wortmannin, and labeled BL-related proteins were analyzed as in *a*. The relative radioactivity in proteins was quantitated using a BAS 2000 image analyzer. □, BL-CTPP; ○, NTPP-BL; ■, I28G-SPO-CTPP.

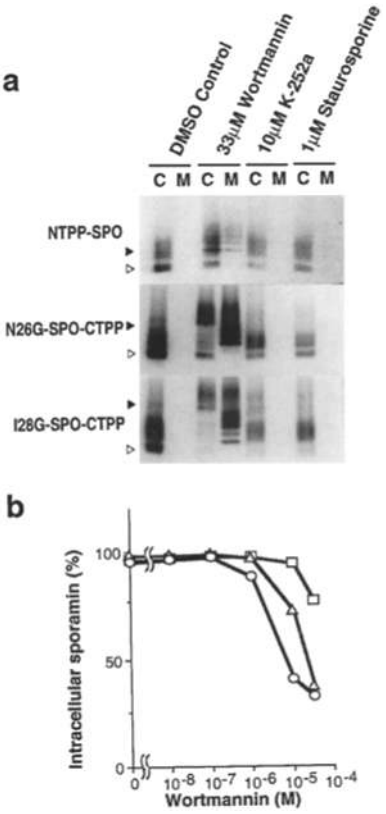

Figure 6. Effects of wortmannin on the transport of wild-type and mutant SPO. (*a*) Transformed tobacco cells were pulse labeled and chased in the presence of wortmannin (33 μM), K-252a (10 μM), or staurosporine (1 μM) as in Fig. 5 *a*, and radiolabeled SPO-related polypeptides were analyzed as in Fig. 4. The solid and open arrowheads indicate the migration position of the precursor and mature forms of the SPO constructs. *C*, cells; *M*, medium. (*b*) Dose-dependent secretion of sporamin constructs in the presence of wortmannin. Transformed tobacco cells were pulse labeled and chased in the presence of wortmannin as in *a* and radiolabeled SPO-related proteins were analyzed as in *a*. The relative radioactivity in proteins was quantitated using a BAS 2000 image analyzer. □, NTPP-SPO; △, N26G-SPO-CTPP; ○, I28G-SPO-CTPP.

240-min chase period (Fig. 5 *a*). Most of the secreted BL-CTPP and I28G-BL-CTPP in the presence of wortmannin migrated as the 18-kD vacuolar form, and only a small amount of the precursor forms were detectable in the medium. This indicates that the CTPPs of these proteins in the medium were processed. The processing of CTPP occurred after the secretion of the precursor, since incubation of the medium after removal of the cells caused a decrease in the precursor form (data not shown). A similar decrease in the relative molecular mass of other proteins with CTPPs in the culture medium of tobacco cells that were treated with wortmannin could be detected (Figs. 6 *a* and 7 *b*).

Less than 20% of the pulse-labeled I28G-SPO-CTPP was converted to the vacuolar form in the cell, and most of the remaining protein was secreted to the medium (Fig. 6 *a*). About 25% of the N26G-SPO-CTPP was converted to the vacuolar form during the chase; however, some of the precursor forms were retained intracellularly (Fig. 6 *a*).

In contrast to the case of the CTPP-containing proteins,

Published September 15, 1995

only small amounts of pulse-labeled NTPP-BL and NTPP-SPO were secreted to the medium in the presence of 33 μM wortmannin, and >70% of the pulse-labeled NTPP-SPO was converted to the vacuolar form during the chase (Figs. 5 *a* and 6 *a*). The IC_{50} values of wortmannin for the sorting of BL-CTPP and I28G-SPO-CTPP were 6.7 and 7.5 μM, respectively, and most of the protein was secreted in the presence of 33 μM wortmannin (Fig. 5 *b*). The wortmannin IC_{50} value for the sorting of NTPP-BL was over 33 μM. These data indicate that sorting to the vacuole by CTPP is more sensitive to wortmannin than that of NTPP and suggest that these two different signals use different machineries for the transport of proteins to the vacuole.

Since wortmannin inhibits not only mammalian PI 3-kinase but also mammalian myosin light chain kinase (Nakanishi et al., 1992), there was a possibility that the inhibition of CTPP-mediated transport by wortmannin is due to the inhibition of protein kinases. To address this possibility, tobacco cells expressing each of the constructs were pulse labeled and chased in the presence of broad-range kinase inhibitors (10 μM K-252a or 1 μM staurosporine), and the inhibition of protein transport was analyzed. About 50% inhibition of protein synthesis was observed in tobacco cells treated with either of the inhibitors (data not shown). However, neither of these two inhibitors caused the secretion of proteins to the medium during the chase periods (Figs. 5 *a* and 6 *a*). These data suggest that the missorting of CTPP-containing proteins by wortmannin is not caused by the inhibition of protein kinases, and that other components are the target of wortmannin in the CTPP-mediated process.

Effect of Wortmannin on the Secretion and Vacuolar Delivery of Endogenous Proteins in Tobacco Cells

To analyze whether the effect of wortmannin is specific for the delivery of proteins by the CTPP of pro-BL, and to address whether wortmannin affects the secretion of proteins from tobacco cells, we first analyzed the change in the pattern of total ^{35}S-labeled proteins in the medium of a tobacco cell culture pulse labeled and chased in the presence of various concentrations of wortmannin (Fig. 7 *a*). In the absence of wortmannin, we observed several major polypeptide bands in the medium (Fig. 7 *a*, lane *1*). In the presence of wortmannin, most of the major bands were detectable in the medium at a similar level as in the absence of wortmannin (Fig. 7 *a*). The kinetics for the secretion of these major proteins were not altered significantly in the presence of wortmannin (data not shown). In contrast, new polypeptide bands of 20, 29, and 33 kD were apparent in the medium with 33 μM wortmannin. It appeared that wortmannin did not affect the transport of most of the secretory proteins in the tobacco cells, but that wortmannin caused the missorting of several intracellular proteins.

To analyze whether wortmannin causes the missorting of tobacco vacuolar proteins other than the SPO- or BL-fusion constructs, we analyzed the effect of wortmannin on the sorting of basic class I chitinase and class I β-1,3-glucanase (Fig. 7, *b* and *c*). Both of these proteins are expressed in tobacco BY-2 cells at a high level (Ohme-Takagi and Shinshi, 1990; Fukuda et al., 1991). Tobacco class I chitinase is synthesized as a preproprotein with an NH_2-terminal signal peptide and CTPP (Neuhaus et al., 1991; Shinshi et al., 1988). It migrates as smeared bands on an SDS–polyacrylamide gel due to proline hydoxylation and the expression of two isoforms from closely related chitinase genes (Sticher et al., 1992, 1993). These proteins are localized in the vacuole of tobacco cells after processing of the CTTPs (Neuhaus et al., 1991). The CTPP of the class I chitinase precursor, which consists of seven amino acids, functions as a vacuolar targeting signal in *Nicotiana* cells (Neuhaus et al., 1991). Although the amino acid composition of this CTPP resembles the CTPP of pro-BL, no conserved amino acid motifs could be found despite a detailed mutational analysis of the pro-BL CTPP (Dombrowski et al., 1993) and the CTPP of the class I chitinase (Neuhaus et al., 1994). The β-1,3-glucanase precursor also has a CTPP containing N-linked glycosylation sites, which are

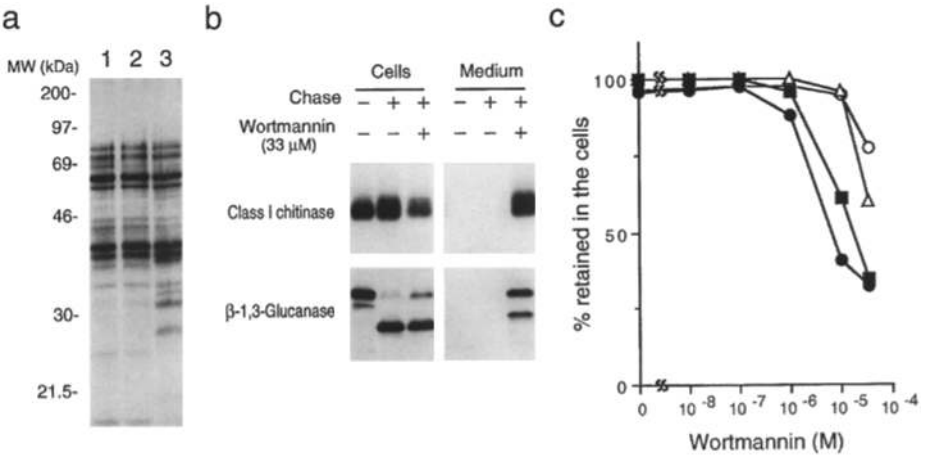

Figure 7. Effect of wortmannin on the transport of secretory and vacuolar proteins other than SPO and BL. (*a*) Effects of wortmannin on the secretion of proteins from tobacco cells. Transformed tobacco cells expressing NTPP-SPO were pulse labeled and chased as in Fig. 6 *b*. Proteins in the medium were recovered by TCA precipitation, separated by SDS-PAGE, and visualized by autoradiography. Lane *1*, without wortmannin; lane *2*, 10 μM wortmannin; lane *3*, 33 μM wortmannin. (*b*) Processing and transport of class I chitinase or β-1,3-glucanase in the absence or presence of wortmannin. Transformed tobacco cell cultures were pulse labeled for 15 min and chased (*chase*) in the absence (−) or presence (+) of 33 μM wortmannin. Class I chitinase and β-1,3-glucanase in the cells or medium were immunoprecipitated by specific antibodies, separated by SDS-PAGE, and visualized by autoradiography. (*c*) Dose dependency for the missorting of class I chitinase and β-1,3-glucanase by wortmannin. Cells were pulse labeled and chased in the presence of wortmannin as in Fig. 5 *b*, and radiolabeled class I chitinase and β-1,3-glucanase were recovered as in *b*. The relative amounts of radioactivity in the precipitated proteins were calculated using a BAS 2000 image analyzer. ○, NTPP-SPO; ●, I28G-SPO-CTPP; ■, class I chitinase; △, β-1,3-glucanase.

Published September 15, 1995

necessary for targeting to the vacuole (Melchers et al., 1993).

In the absence of wortmannin, pulse-labeled chitinase was retained intracellularly after a 240-min chase. In the presence of 33 μM wortmannin, >70% of pulse-labeled chitinase was secreted to the medium (Fig. 7 *b*, *chitinase*). The missorting of chitinase caused by wortmannin shows a similar dose dependency to that of I28G-SPO-CTPP (Fig. 7 *c*), suggesting that the vacuolar delivery of chitinase by its CTPP uses a similar mechanism to that of the BL CTPP. The β-1,3-glucanase precursor that was detectable in pulse-labeled tobacco cells migrated as 40-kD and 38-kD polypeptide bands on an SDS gel (Fig. 7 *b*; data not shown), and these precursors were converted to 32-kD mature glucanase during the chase periods. In the presence of 33 μM wortmannin, ~30% of pulse-labeled glucanase was secreted to the medium, with >60% of the labeled glucanase converted to the vacuolar form in the cell (Fig. 7 *b*). The transport of glucanase showed an intermediate sensitivity to wortmannin between the NTPP- and CTPP-mediated transport of SPO to the vacuoles (Fig. 7 *c*).

Effect of Wortmannin on the Activity of PI 3-Kinase in Tobacco Cells

Low nanomolar concentrations of wortmannin inhibit mammalian PI 3-kinase (Yano et al., 1993; Arcaro and Wymann, 1993), but a homologous yeast PI 3-kinase is not inhibited by this reagent at this concentration (Woschloski et al., 1994). To address whether the inhibition of CTPP-mediated vacuolar sorting by wortmannin is due to the inhibition of PI 3-kinase activity in tobacco cells, we analyzed the effect of wortmannin on tobacco cell PI 3-kinase activity (Fig. 8). A total tobacco cell homogenate was incubated with PI and γ-[^{33}P]ATP, and the phospholipids in the reaction mixture were separated using a borate TLC system. A yeast membrane fraction was used as a control for the formation of PI 3-phosphate, since it contains high PI 3-kinase activity (Schu et al., 1993). Two spots of ^{33}P-labeled PI phosphates migrating with the same Rf values as those of the yeast membrane fraction were produced by the tobacco cell lysate (Fig. 8 *a*, lanes *1* and *2*). To confirm that the spots are ^{33}P-labeled PI phosphates, lipids were extracted from these spots, separated by alkaline TLC, and the Rf values compared with authentic PI 4-phosphate. Although this TLC system cannot separate PI 3-phosphate and PI 4-phosphate efficiently, this system can separate PI phosphates from other phospholipids and lysophospholipids (Fig. 9 *a*; Munnik et al., 1994). Both of the ^{33}P-labeled lipids gave single spots on alkaline TLC and migrated to the same position as authentic PI 4-phosphate, indicating that these spots are PI phosphates (data not shown). These data and the Rf values on borate TLC plates indicated that the upper of the two spots is PI 3-phosphate and the lower is PI 4-phosphate, and that both PI 3-kinase and PI 4-kinase activities are present in tobacco cells. To investigate the intracellular localization of the PI 3-kinase, soluble and membrane fractions were prepared from the tobacco cell lysate and the PI kinase activity analyzed. Most of the PI 3-kinase activity was recovered in the membrane fraction (Fig. 8 *a*, lane *4*), with <10% of the activity in the soluble fraction (Fig. 8 *a*, lane *5*). In contrast, PI 4-kinase activity distributed almost equally between the

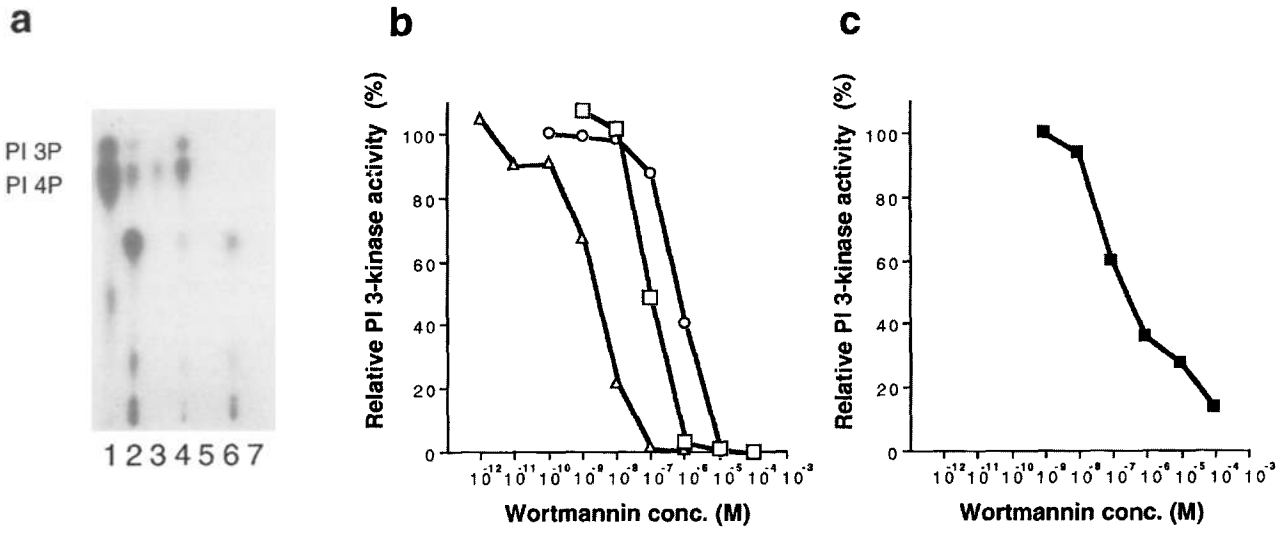

Figure 8. Inhibition of PI kinase activities by wortmannin. (*a*) Tobacco cell PI 3-kinase and PI 4-kinase were almost completely inhibited in the presence of 33 μM wortmannin. TLC of PI kinase assay reaction products preformed with the S1, P100, and S100 fractions isolated from tobacco BY-2 cells in the absence and presence of wortmannin at 33 μM are shown. A yeast cell lysate was used to generate reference markers for PI 3-phosphate and PI 4-phosphate. Lane *1*, yeast cell lysate; lane *2*, tobacco S1; lane *3*, tobacco S100; lane *4*, tobacco P100; lane *5*, tobacco S1 with 33 μM wortmannin; lane *6*, tobacco S100 with 33 μM wortmannin; lane *7*, tobacco P100 with 33 μM wortmannin. (*b*) Effects of wortmannin on the activities of mammalian, yeast, and tobacco PI 3-kinase in vitro. To determine inhibition of PI 3-kinase in vitro, PI 3-phosphate formation was measured in the presence of the indicated concentration of wortmannin. △, rat spleen in vitro; ○, yeast in vitro; □, tobacco cell in vitro. (*c*) Effect of wortmannin on the activity of tobacco PI 3-kinase in vivo. Inhibition of PI 3-kinase activity in tobacco cells in vivo was analyzed by measuring the activity of a tobacco cell lysate after preincubation of the cells with the indicated concentrations of wortmannin for 45 min at 28°C.

Published September 15, 1995

soluble and membrane fractions. When the activities of tobacco cell PI kinases were assayed in the presence of 33 μM wortmannin, both PI 3-kinase and PI 4-kinase activities were almost completely inhibited (Fig. 8 *a*, lanes 5–7). The dose dependency for the inhibition of tobacco PI 3-kinase by wortmannin was compared with that of rat spleen and yeast PI 3-kinases (Fig. 8 *b*). In the presence of 1 μM wortmannin, the activity of tobacco PI 3-kinase was inhibited >95% (Fig. 8 *b*, tobacco cell in vitro). An IC_{50} value of 98 nM wortmannin was calculated for tobacco cell PI 3-kinase. An IC_{50} value of 4.1 nM wortmannin was determined for rat spleen PI 3-kinase under our assay conditions (Fig. 8 *b*, rat spleen), similar to that observed for calf thymus PI 3-kinase (Yano et al., 1993). The IC_{50} value for yeast PI 3-kinase was 930 nM. Therefore, tobacco cell PI 3-kinase displayed characteristics in between mammalian and yeast PI 3-kinases with respect to wortmannin sensitivity. The IC_{50} value for tobacco cell PI 3-kinase was much lower than that for the sorting of CTPP-containing proteins. This can be explained if wortmannin cannot efficiently inhibit the PI 3-kinase in tobacco cells in vivo, or if another target of wortmannin (other than PI 3-kinase), which is involved in CTPP-mediated sorting events, exists in tobacco cells. To distinguish between these possibilities, we prepared cell lysates from tobacco cells treated with various concentrations of wortmannin for 60 min and analyzed the dose dependency of inhibition of PI 3-kinase activity by wortmannin in vivo (Fig. 8 *c*, in vivo). The IC_{50} value in vivo was calculated to be 700 nM, but the PI 3-kinase activity was not inhibited completely even when the tobacco cells were incubated for longer periods of up to 150 min with 100 μM wortmannin, or by the addition of wortmannin repeatedly at 10-min intervals (data not shown). The dose dependency for the inhibition of PI 3-kinase in vivo did not correspond to that of the missorting of CTPP-containing proteins, with higher concentrations of wortmannin required for inhibition of vacuolar protein targeting (Figs. 5 *b*, 6 *b*, 7 *c*, and 8 *c*).

Wortmannin Inhibits the Synthesis of Phospholipids with a Similar Dose Dependency as That for the Inhibition of CTPP-mediated Transport

The data presented in Figs. 5–8 suggest that the inhibition of PI 3-kinase or protein kinase in tobacco cells is not responsible for the missorting of the CTPP-containing proteins. To address whether the missorting of these proteins by wortmannin correlates with an effect on PI metabolism, we analyzed the effect of wortmannin on the synthesis of PI and other phospholipids in tobacco cells. Tobacco cells were labeled with carrier-free [^{32}P]phosphate for 90 min in the absence and presence of various concentrations of wortmannin, then phospholipids were extracted from the labeled cells and separated by alkaline TLC or borate TLC systems. For up to 90 min of labeling, the incorporation of ^{32}P into phospholipids or PI phosphates was proportional to the labeling time (data not shown); thus we used 90-min labeling for the analysis of the synthesis of phospholipids. The radioactivity in the phospholipid spots was quantitated using a BAS 2000 image analyzer (Fig. 9 *a*).

The inhibitory effect of wortmannin on the synthesis of PI 3-phosphate was not consistent with the inhibition of PI 3-kinase activity in vivo (Fig. 8 *c* and 9 *b*, *inset*). The IC_{50} value of wortmannin for the synthesis of PI 3-phosphate was calculated to be 1.1 μM, and this value is lower than those for the sorting of CTPP-containing proteins (~7 μM). In the presence of micromolar concentrations of wortmannin, the synthesis of most phospholipids, including PI, phosphatidylcholine, phosphatidic acid, and PI 4-phosphate, was affected, and the IC_{50} values for the synthesis of these phospholipids were ~9 μM (Fig. 9 *b*). The inhibition of synthesis is not due to a general inhibition of ATP synthesis (data not shown). This dose dependency is approximately equal to the dose dependency for the inhibition of CTPP-mediated vacuolar delivery, suggesting that phospholipid synthesis could be involved in the mechanism of CTPP-mediated vacuolar sorting in tobacco cells.

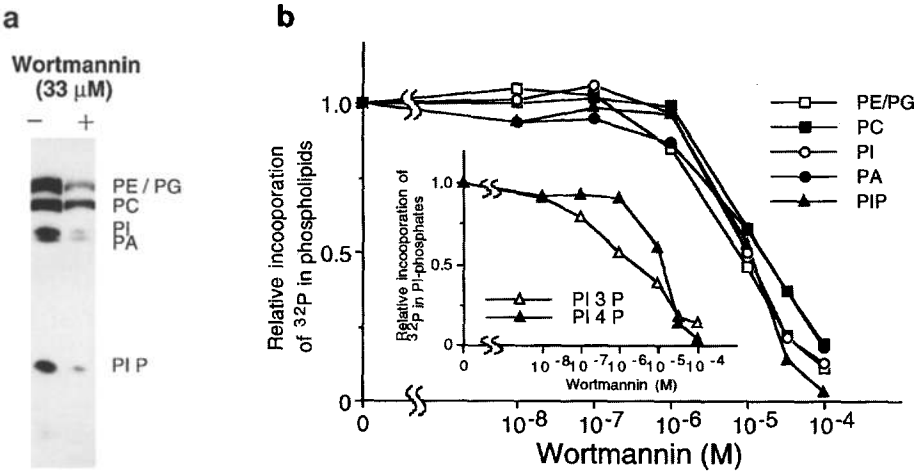

Figure 9. Effects of wortmannin on the synthesis of phospholipids in tobacco cells. (*a*) TLC of radiolabeled phospholipids in tobacco cells in the absence and presence of 33 μM wortmannin during 90-min pulse labeling. The migration position of phosphatidylethanolamine and phosphatidylglycerol (*PE/PG*), phosphatidylcholine (*PC*), PI (*PI*), phosphatidic acid (*PA*), and PI phosphates (*PIP*) are indicated. (*b*) Dose-dependent inhibition of phospholipid synthesis by wortmannin. Phospholipids in tobacco cells were pulse labeled as in *a* in the presence of the indicated concentration of wortmannin. Lipids were extracted and separated by alkaline TLC or borate TLC (*inset*). The amount of radioactivity of each of the phospholipids relative to the no-inhibitor control is shown.

Published September 15, 1995

Discussion

Most of the newly synthesized soluble proteins that accumulate in plant and yeast vacuoles and mammalian lysosomes are sorted from the secretory pathway to the vacuolar (lysosomal) transport pathway at the *trans*-Golgi network. Sorting of plant vacuolar proteins from secretory proteins requires specific targeting signals within the structure of these proteins (Chrispeels and Raikhel, 1992). To date, signals for sorting to the vacuole in plant cells have been identified in several proteins, such as sweet potato SPO, BL, tobacco class I chitinase, barley aleurain, and bean phytohemagglutinin and legumin (Bednarek et al., 1990; Bednarek and Raikhel, 1991; Matsuoka and Nakamura, 1991; Neuhaus et al., 1991; Saalbach et al., 1991; Holwerda et al., 1992; Nakamura et al., 1993; von Schaewen and Chrispeels, 1993). In the case of prosporamin, an Asn-Pro-Ile-Arg sequence in the NTPP of the precursor functions as a vacuolar targeting signal, and a similar sequence can be found in the NTPP of the aleurain precursor and some of the putative propeptides of potato tuber proteins (Chrispeels and Raikhel, 1992; Nakamura and Matsuoka, 1993; Ishikawa et al., 1994). The BL CTPP functions as a vacuolar targeting signal (Bednarek et al., 1990; Bednarek and Raikhel, 1991), as does the CTPP of class I chitinase in tobacco (Neuhaus et al., 1991). No obvious sequence similarities between the CTPPs of BL and chitinase were found, or between CTPPs and NTPPs, although SPO and BL are transported to the same vacuoles when they are coexpressed in tobacco (Schroeder et al., 1993).

To examine whether NTPP- and CTPP-mediated vacuolar delivery occurs by the same mechanism in tobacco cells, we first analyzed whether the sporamin and BL propeptides are interchangeable. Although the structures of BL and sporamin are thought to be very different (Onesti et al., 1991; Chrispeels and Raikhel, 1992; Ishikawa et al., 1994), both of these proteins are targeted to the vacuole by both NTPP and CTPP (Figs. 2–4). In addition, the rate of transport to the vacuole is dependent on the protein being transported, not on the targeting signal used.

Our previous analysis of the coexpression of BL-CTPP and NTPP-SPO in the same tobacco plant (Schroeder et al., 1993) indicated that these proteins are correctly sorted to the same vacuole within cells, with no saturation of the sorting machinery seen despite relatively high levels of expression. In addition, a peptide consisting of the prosporamin NTPP was able to compete with the propeptide of proaleurain for binding to a putative vacuolar targeting receptor, whereas the pro-BL CTPP could not compete (Kirsch et al., 1994). The fact that the sensitivity to wortmannin is similar for the sorting of most proteins containing the CTPP of BL and for class 1 chitinase (Fig. 7, *b* and *c*) suggests that these proteins share similar mechanisms for vacuolar sorting. In contrast, CTPP- and NTPP-mediated vacuolar delivery show differential sensitivities to wortmannin (Figs. 5 and 6), suggesting that they use different targeting mechanisms. It is possible that the differential sensitivities to wortmannin may be due to structural differences between the two types of targeting signal, rather than the difference in the location of the sorting signals. Thus, it appears that (some) plant cells have two or more machineries for the delivery of soluble proteins to the vacuole, and at least one of them is sensitive to wortmannin.

In mammalian cells, most of the soluble proteins in the lysosomes are sorted from the secretory pathway by a signal consisting of a mannose-6-phosphate residue, while membrane proteins use different sorting mechanisms (von Figura and Hasilik, 1986). In plant cells it was previously shown that the delivery of soluble and membrane proteins to the vacuole occurs via different pathways (Gomez and Chrispeels, 1993). An alternative, Golgi-independent pathway to the vacuole has been proposed to operate in the case of some seed storage proteins, where protein bodies derived from the ER are directly engulfed by the vacuole (Galili et al., 1993). In addition, the data presented here indicate that plant cells use at least two different machineries for the sorting of soluble proteins to the vacuole. BL and class I chitinase use the wortmannin-sensitive mechanism, while sporamin uses the wortmannin-resistant pathway. It is presently not known whether proteins containing different types of targeting signal are carried to the vacuole in the same vesicles. The existence of a putative receptor specific for one class of targeting signal (Kirsch et al., 1994), and the presence of multiple targeting mechanisms (this work), opens the possibility of subsets of transport vesicles responsible for the delivery of proteins containing a particular class of sorting signal.

The dose dependencies for the inhibition of PI 3-kinase by wortmannin and the synthesis of PI 3-phosphate in tobacco cells were different than that for the disruption of CTPP-mediated vacuolar delivery. However, our data do not rule out the possibility of the involvement of PI 3-kinase or PI 3-phosphate in the delivery and accumulation of vacuolar proteins or the organization of endomembrane organelles in plant cells. Hong and Verma (1994) recently showed that a specific PI 3-kinase is induced during soybean nodule formation. During the development of nodules, a specific membrane called the peribacteroid membrane, which has characteristics of both the plasma membrane and vacuolar membrane, develops extensively. The expression of a specific PI 3-kinase occurs concomitant to the formation of the peribacteroid membrane during nodule development, and the PI 3-kinase could contribute to the membrane organization during nodule development (Hong and Verma, 1994). In *Arabidopsis thaliana*, it was recently shown that a PI 3-kinase is essential for development (Welters et al., 1994). In addition, the conditions used in this study for the treatment of tobacco cells with wortmannin did not decrease the steady state level of PI 3-phosphate significantly (Matsuoka, K., unpublished results). These findings, and the involvement of PI 3-kinase in vesicular trafficking in yeast (Schu et al., 1993; Stack et al., 1993) and mammalian cells (Yano et al., 1993; Joly et al., 1994; Kotani et al., 1994) indicate that further study is necessary to elucidate the involvement of PI 3-phosphate in membrane organization and vacuolar delivery of proteins in tobacco cells.

The treatment of tobacco cells with wortmannin inhibited not only PI 3-kinase but also PI 4-kinase and the synthesis of phospholipids (Fig. 9), and the dose dependency for the inhibition of the synthesis of PI 4-phosphate and

Published September 15, 1995

phospholipids was similar to the dose dependency for the disruption of CTPP-mediated delivery to the vacuole. In yeast cells, it was reported that maintaining the ratio of PI/phosphatidylcholine in the Golgi apparatus is essential for the secretion of proteins from the Golgi apparatus, and Sec14p is essential for maintenance of this ratio (McGee et al., 1994). It is possible that the treatment of tobacco cells with wortmannin inhibits the synthesis of phospholipids, causing an alteration in the phospholipid composition of the Golgi apparatus, and hence it may affect the sorting of proteins to the vacuole. Another possibility is that the disruption of CTPP-mediated vacuolar sorting is due to the inhibition of PI 4-kinase, and therefore the formation of PI 4-phosphate. Preliminary analysis on the synthesis and turnover of PI-phosphate in tobacco cells indicates that the PI 4-kinase in tobacco cells is not only associated with the plasma membrane in several plants (reviewed in Drøbak, 1992) but also associated with the Golgi apparatus, and upon treatment with wortmannin the level of PI 4-phosphate decreased rapidly (Matsuoka, K., unpublished results). In addition, PI 4-kinase has been suggested to be involved in intracellular protein traffic in mammalian cells (Del Vecchio and Pilch, 1991). It is possible that the PI 4-phosphate that is synthesized in the Golgi apparatus may change the curvature of the membrane as discussed for PI 3-phosphate in yeast cells (Stack et al., 1993). Alternatively, PI 4-phosphate and other phospholipids may regulate the membrane association of proteins participating in sorting, or these phospholipids may activate the sorting apparatus. It was observed in mammalian cells that dynamin, a GTPase involved in endocytosis (van der Bliek et al., 1993; Herskovits et al., 1993), is activated by acidic phospholipids (Tuma et al., 1993). More analysis is therefore necessary to elucidate the target of wortmannin in tobacco cells in order to understand the sorting of proteins to the vacuole.

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