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Protein Sorting to the Vacuolar Membrane

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The vacuolar membrane (tonoplast) of plant cells contains a polytopic integral membrane protein with six membrane-spanning domains and cytoplasmically oriented amino-terminal and carboxy-terminal domains. This protein, tonoplast intrinsic protein (TIP), is a member of the membrane intrinsic protein (MIP) family of proteins, a family of channel proteins found in a variety of organisms. In bean seeds, α-TIP is synthesized on the rough endoplasmic reticulum and its transport to the tonoplast is mediated by the secretory system. In this study, we report that a polypeptide segment that includes the sixth membrane domain and the cytoplasmic tail of 18 amino acids of α-TIP is sufficient to target the reporter protein phosphinotricine acetyltransferase to the tonoplast of stably transformed tobacco cells. To determine if the carboxy-terminal cytoplasmic tail of α-TIP contains important tonoplast targeting information, a deletion construct lacking the 15 carboxy-terminal amino acids was introduced for transient expression in tobacco cells; we found that the slightly truncated protein still accumulated in the tonoplast. From these results, we concluded that a transmembrane domain of a tonoplast protein probably contains sufficient information for transport to the tonoplast. Whether such transport occurs by bulk flow or involves specific cellular machinery remains to be determined.

INTRODUCTION

The secretory system of eukaryotic cells is the site of synthesis, modification, and sorting of proteins that are secreted, accumulate in vacuoles, or are retained in various compartments of the endomembrane system itself. The secretory system sorts soluble proteins, as well as membrane proteins, delivering them not only to the vacuole and the cell wall, but also to the plasma membrane and the tonoplast (vacuolar membrane). Recent results from several laboratories have led to a better understanding of the information required for the sorting of soluble proteins in the secretory system of plant cells. After sequestration in the lumen of the endoplasmic reticulum (ER), soluble proteins may be retained there by specific retention signals, such as the carboxy-terminal tetrapeptide KDEL, secreted by a bulk flow or default pathway, or sorted to the vacuole (for a review, see Chrispeels, 1991). Short peptide domains have recently been identified in several vacuolar proteins that are both necessary and sufficient for vacuolar targeting (for a review, see Chrispeels and Raikhel, 1992).

There is, as yet, no information about the sorting in plant cells of the membrane proteins of the secretory system. Many membrane proteins are glycoproteins, and the presence of complex glycans on both plasma membrane and tonoplast proteins implies that the secretory system sorts and delivers these proteins to their respective destinations because the formation of complex glycans occurs in the Golgi apparatus (for a review, see Faye et al., 1989). In mammalian cells, synthesis of intrinsic membrane proteins takes place on the ER, and transport to the plasma membrane occurs by default, unless the proteins are held back by specific structural motifs (for a review, see Pfeffer and Rothman, 1987). Such retention information can be present in the transmembrane domain, as it is for Golgi localized proteins such as the enzymes β-1,4-galactosyltransferase (Nilsson et al., 1991) and α-2,6-sialyltransferase (Munro, 1991) and coronavirus E1 protein (Machamer and Rose, 1987). Sorting to the lysosomal membrane is a complex process in mammalian cells and involves not only the ER and the Golgi, but also the plasma membrane and the endosomes. For example, lysosomal acid phosphatase, a typical lysosomal membrane protein, is first transported to the plasma membrane via the Golgi apparatus and then internalized by endocytosis. Transport from the plasma membrane to the lysosomal membrane requires an endocytosis signal in the short cytoplasmic tail of the protein (Peters et al., 1990).

The amino acid sequences of only a few plasma membrane and tonoplast proteins of plants have been determined, and there is, as yet, no information on their transport or targeting. In this paper, we report the identification of a short polypeptide domain of tonoplast intrinsic protein (TIP) that may be involved in targeting this protein to the tonoplast. TIP is an abundant 27-kD protein that has six membrane-spanning domains and is present in different isoforms in embryos (seeds) and vegetative tissues (Johnson et al., 1990; Höfte et al., 1992). The protein is synthesized on the rough ER before it is transported to the vacuolar membrane (Mäder and Chrispeels,

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1984). We have analyzed tonoplast targeting information in TIP and have found that a polypeptide segment comprising the sixth membrane-spanning domain and the cytoplasmic tail of TIP contains sufficient targeting information to allow a protein to be transported to the tonoplast. In addition, the cytoplasmic tail is not necessary for tonoplast targeting. What is not yet clear is whether such transport occurs by bulk flow or requires specific cellular targeting machinery.

RESULTS

Time Course of TIP Transport

The integral tonoplast protein α-TIP is synthesized on the rough ER of bean cotyledons (Mäder and Chrispeels, 1984) and passes through the Golgi apparatus (Melroy and Herman, 1991) on its way to the tonoplast. This protein, therefore, follows the same transport route as the soluble proteins that are secreted or accumulate in vacuoles. We recently developed a heterologous expression system for α-TIP using transgenic tobacco plants and showed that, in tobacco leaves, α-TIP was targeted to the tonoplasts of the large vacuoles in mesophyll cells (Höfte et al., 1991). As a prelude to identifying tonoplast targeting information in α-TIP, we examined the kinetics of its transport in transgenic tobacco cells in which the expression of α-TIP is driven by the cauliflower mosaic virus (CaMV) 35S promoter. Protoplasts obtained from the leaves of these transgenic plants were labeled with ^{35}S-methionine and ^{35}S-cysteine (Express 35) for 1 hr, and the radioactivity was chased with unlabeled amino acids for different time periods. Vacuoles were prepared from the protoplasts, and their acid phosphatase content was determined and used as a guide for the quantitation of the amount of protoplast or vacuolar material to be loaded in each lane of an SDS–polyacrylamide gel. Radioactive α-TIP was immunoprecipitated with α-TIP antiserum and analyzed by SDS-PAGE and fluorography, as shown in Figure 1. A sample of α-TIP immunoprecipitated from total protoplasts, labeled for 1 hr and with the same amount of acid phosphatase activity, served as a control for the total amount of labeled α-TIP synthesized by the cells and is shown in the first lane of Figure 1. The purity of the vacuole preparation was confirmed by the absence of the lumenal ER marker binding protein (BiP) (data not shown here, but see below for a similar control) in immunoprecipitates with anti-BiP antibodies. Small amounts of α-TIP could already be detected in the purified vacuoles after 1 hr of labeling (Figure 1, lane 1). After 6 hr of chase, the α-TIP signal in the vacuole fraction equaled the signal from the protoplasts, indicating that all the radioactively labeled α-TIP had arrived in the tonoplast after 6 hr.

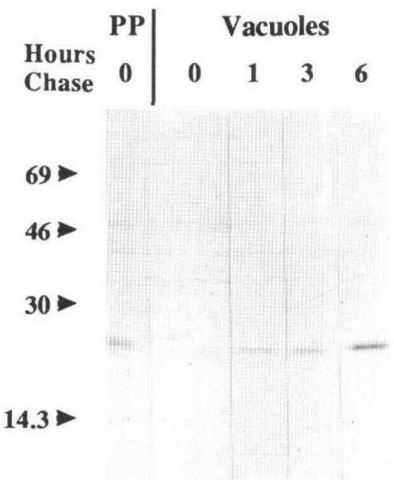

Figure 1. Accumulation of Radioactive TIP in the Vacuoles of Protoplasts Prepared from Transgenic Tobacco Leaves.

Fluorograph of α-TIP immunoprecipitated from protoplast (PP) and vacuolar extracts containing the same amount of the vacuolar marker enzyme acid phosphatase. Protoplasts were labeled for 1 hr with ^{35}S-labeled amino acids, and the radioactivity was chased for 0, 1, 3, and 6 hr. Note that the vacuole fraction after 6 hr of chase contains approximately the same amount of radioactive α-TIP as the total protoplasts. Arrowheads refer to the position of the molecular size standards in kilodaltons.

PAT-TIP: A Fusion between a Soluble Secreted Protein and 47 Carboxy-Terminal Amino Acids of TIP Is an Integral Membrane Protein

Studies with animal cells that have been reviewed by Hopkins (1992) demonstrate that the targeting of integral membrane proteins to the lysosome (the vacuolar homolog in animal cells) is mediated by a peptide sequence present in the cytoplasmically oriented carboxy terminus. For instance, a lysosomal protein (h-lamp-1) in which this signal had been disrupted accumulated at the cell surface, the default destination. A cell surface protein, on the other hand, could be redirected to the lysosome through the addition of such a peptide domain (Williams and Fukuda, 1990). Although these studies were carried out on type I membrane proteins with only one transmembrane domain, we considered it important to investigate whether similar vacuolar targeting signals could be identified in the polytopic membrane protein α-TIP.

Our approach was to study the intracellular fate of a fusion protein consisting of the chimeric secreted protein ssPAT (signal sequence–phosphinotricine acetyltransferase) (Denecke et al., 1990) and the carboxy-terminal 48 amino acids of α-TIP, as shown in Figure 2. PAT is a *Streptomyces hygroscopicus* enzyme that acetylates and inactivates the herbicide phosphinotricin. Denecke et al. (1990) previously showed that this protein was secreted from transgenic tobacco cells when fused to a signal peptide derived from the pathogenesis-related protein 1 (PR-1) of tobacco. Moreover, the signal peptide was correctly cleaved from the enzyme during this process. After confirming the results of Denecke et al. (1990) in our laboratory

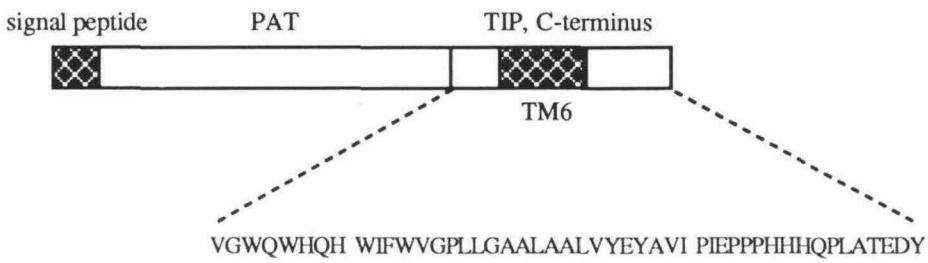

Figure 2. Schematic Representation of the Fusion Protein ssPAT-TIP Expressed in Transgenic Tobacco Cells.

The amino acid sequence refers to the portion of the fusion protein derived from α-TIP (the carboxy-terminal 48 amino acids). The shaded area toward the carboxy terminus refers to the sixth predicted transmembrane sequence (TM6) of α-TIP.

(see below), we used the same ssPAT construct used by Denecke et al. (1990) and fused it to the carboxy terminus of α-TIP.

The α-TIP–derived portion of the PAT-TIP fusion protein contained a sequence of 23 hydrophobic amino acids characteristic of a membrane-spanning domain—the sixth predicted transmembrane domain of α-TIP (TM6)—followed by a stretch of 17 hydrophilic amino acids that, based on structure predictions, have a cytoplasmic orientation (Figure 2; Johnson et al., 1990). We reasoned that TM6 could serve as a membrane anchor and convert the soluble reporter protein PAT into an integral membrane protein. It is unlikely that PAT itself contains any targeting information, because it is secreted into the extracellular medium—the default pathway—when introduced in the secretory system of plant cells. Therefore, the intracellular localization of the fusion protein could tell us whether or not the α-TIP–derived fragment contained any tonoplast targeting information.

As a control experiment, we generated transgenic tobacco suspension culture cells (tobacco cells) expressing ssPAT from the CaMV 35S promoter. The results, shown in Figure 3, demonstrate that the chimeric protein was indeed synthesized in protoplasts prepared from these cells and secreted into the culture medium. A single radioactive band corresponding to the mature PAT protein was observed, indicating that the signal sequence was efficiently removed after import into the rough ER. When protoplasts were labeled with ^{35}S-labeled amino acids for 1 hr and the radioactivity chased for 1, 3, 5, and 7 hr, PAT protein was detected in the protoplasts and in the culture medium (Figure 3). Interestingly, significantly less labeled PAT accumulated in the culture medium compared to the amount of protein initially synthesized by the cells (compare the intensity of the radioactive PAT present in the culture medium at the 7-hr chase and in the protoplasts at time 0). Because PAT is stable in the culture medium (Denecke et al., 1990; H. Höfte and M. J. Chrispeels, unpublished data), this suggests that a substantial portion of the protein is broken down during the intracellular transport.

Next, transgenic tobacco cells were generated expressing the PAT-TIP fusion protein from the same promoter. When these cells were labeled and chased for 1 and 6 hr, respectively, no fusion protein could be detected in immunoprecipitates of the cell culture medium; all the labeled PAT-TIP remained inside the cell, as shown in Figure 4. The same results were obtained when protoplasts were used instead of intact cells (data not shown). To find out if the PAT-TIP that remained in the cells was anchored in the membranes, we extracted the membrane fraction with 100 mM Na_2CO_3, pH 11.5; this is a procedure used to distinguish integral from peripheral membrane proteins. All the PAT-TIP remained associated with the Na_2CO_3-extracted membranes (Figure 4), confirming that the chimeric protein was an integral membrane protein.

To assess the stability of the fusion protein, transformed cells were labeled for 1 hr, and the radioactivity was chased for 2.5, 9, or 19.5 hr before extraction and immunoprecipitation. The labeling pattern of PAT-TIP, as indicated in Figure 5, showed that no significant degradation of the protein occurred during the time span considered, indicating that the half-life is substantially longer than 19.5 hr. Unlike PAT, PAT-TIP appears not to be broken down in the secretory system.

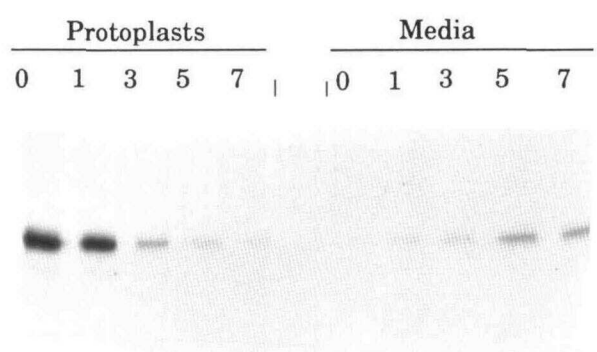

Figure 3. Secretion of PAT in the Culture Medium of Protoplasts Prepared from Transgenic Tobacco Suspension Cells.

Fluorograph of PAT immunoprecipitated from protoplasts and from the culture medium (Media). Protoplasts prepared from transgenic tobacco cells were labeled with ^{35}S-labeled amino acids for 1 hr, and the radioactivity was chased for 0, 1, 3, 5, and 7 hr. Equivalent amounts of protoplasts and media aliquots were loaded in each lane.

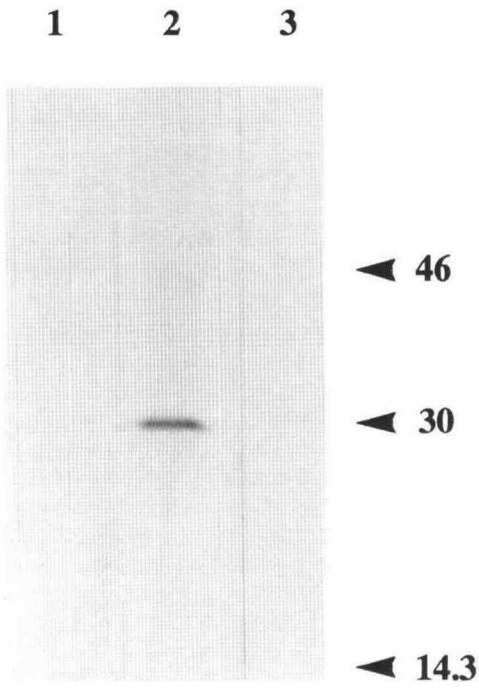

Figure 4. Localization of PAT-TIP in the Integral Membrane Protein Fraction of Transgenic Tobacco Cells.

Fluorograph of PAT-TIP immunoprecipitated from the culture medium (lane 1), from the microsomal pellet after Na_2CO_3 extraction (lane 2), and from the Na_2CO_3 extract of the microsomes (lane 3). Transgenic tobacco cells were labeled with ^{35}S-labeled amino acids for 1 hr, and the radioactivity was chased for 6 hr. Cells were lysed, and microsomes were extracted with 100 mM Na_2CO_3, a procedure that separates intrinsic from peripheral membrane proteins. Note that all of the PAT-TIP protein remained in the Na_2CO_3-extracted membranes, indicating that PAT-TIP is an intrinsic membrane protein. Molecular size markers at right are given in kilodaltons.

PAT-TIP Is a Vacuolar Protein

To determine the intracellular location of PAT-TIP, protoplasts were labeled for 1.5 hr and chased for 5 hr, and the vacuoles were then isolated. First, to assess the purity of the vacuole preparation, 30 μg of protoplast proteins and a sample of the vacuoles containing the same amount of acid phosphatase activity as the protoplast sample were analyzed on a Coomassie Brilliant Blue R 250–stained polyacrylamide gel. As shown in Figure 6A, the protoplast sample showed a multitude of protein bands, whereas in the vacuole sample we could practically only detect BSA, the carrier protein added during the purification (see Methods). Immunoblotting of the same samples with antibodies against the lumenal ER protein BiP confirmed the absence of detectable ER contamination in the vacuole preparation (Figure 6B). To find out if PAT-TIP was in the vacuole fraction, we immunoprecipitated the chimeric radioactive protein with a mixture of antibodies raised against PAT and α-TIP. Figure 7 shows the presence of similar amounts of the labeled protein in the purified vacuoles and in the total protoplasts, indicating a vacuolar localization for PAT-TIP. In addition, because no significant breakdown of the protein occurred during the time span of the experiment (Figure 5), we concluded that most, if not all, synthesized PAT-TIP was transported to the vacuoles. Because PAT-TIP is an integral membrane protein (see above), we presume that it is associated with the tonoplast.

Orientation of PAT-TIP in the Tonoplast

Membrane proteins that have a single membrane-spanning domain can adopt a type I or II orientation. Type I proteins have their carboxy terminus in the cytoplasm, whereas type II proteins have their amino terminus in the cytoplasm. We presume that PAT-TIP is inserted into the ER membrane by means of the signal peptide and is retained in the membrane by the α-TIP–derived membrane-spanning sequence. The fusion protein could theoretically adopt a type I or II orientation within the membrane, as shown in Figure 8A. However, for the study of potential targeting signals within α-TIP, it is essential that the protein have the same orientation within the membrane as the corresponding portion in α-TIP itself. This orientation is predicted to be that of a type I protein, which means that the bulk of the PAT-TIP protein should be present in the lumen with only a small polypeptide portion (17 carboxy-terminal amino acids) exposed to the cytoplasm.

To investigate the PAT-TIP orientation, vacuoles purified from transgenic tobacco cells were incubated with proteinase K either in the absence or presence of detergent, and PAT-TIP was detected after SDS-PAGE by immunoblotting with anti-PAT antibodies (Figure 8B). PAT-TIP was slightly reduced in size after

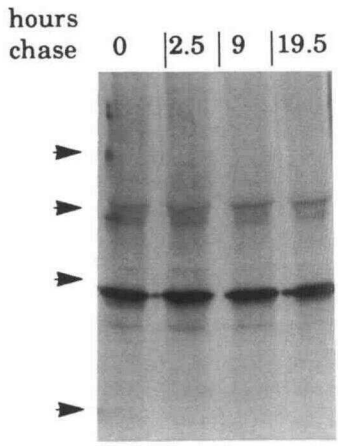

Figure 5. Stability of PAT-TIP in Transgenic Tobacco Cells.

Fluorograph of PAT-TIP immunoprecipitated from lysates of transgenic tobacco cells. Tobacco cells were labeled with ^{35}S-labeled amino acids for 1 hr, and the radioactivity was chased for 0, 2.5, 9, and 19.5 hr, respectively. Molecular size markers are as given in Figure 1.

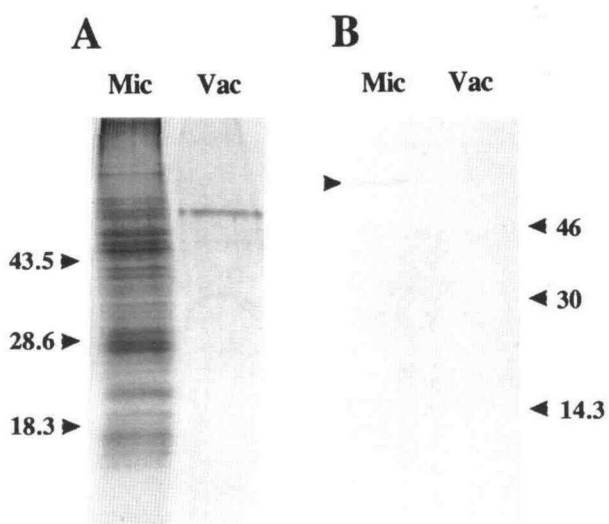

Figure 6. Assessment of the Purity of the Vacuole Preparation from Transgenic Tobacco Cells.

(A) Coomassie blue–stained gel of protein extracts of microsomes and vacuoles containing the same amount of activity of the vacuolar marker enzyme acid phosphatase. The major stained band in the vacuolar fraction is BSA (66.2 kD), added during the vacuole isolation procedure.
(B) Immunoblot of the same samples with an antiserum against the ER resident protein BiP.
Total microsomes (Mic) and vacuoles (Vac) were prepared from tobacco cells transgenic for the ssPAT-TIP gene. The arrowhead in the center indicates the position of BiP.
Molecular size markers at left and right are given in kilodaltons.

treatment of the vacuoles with proteinase K, consistent with the removal of only the 17 carboxy-terminal amino acids. However, this result does not rule out the alternate interpretation that a small fragment of PAT-TIP was removed from the amino terminus. The protein was further degraded by proteinase K in the presence of detergent, although not completely, even with twice the amount of protease. The incomplete degradation of PAT-TIP in the presence of detergent suggests that PAT is a highly protease-resistant protein, consistent with the observation that PAT-TIP is only slowly degraded even in the protease-rich environment within the vacuole (Figure 5). The further degradation of PAT-TIP in the presence of a nondenaturing detergent suggests that the major portion of the protein is inside the membrane vesicle.

Immunoblotting of the same samples with anti–α-TIP antibodies showed that protease treatment removed all cross-reacting material even in the absence of detergent (data not shown). Our polyclonal antiserum to α-TIP contains antibodies that recognize different epitopes on both sides of the membrane (Melroy and Herman, 1991). In the PAT-TIP construct, only the carboxy-terminal portion of TIP is present, and the results described above indicate that there are antibodies to the carboxy-terminal cytoplasmic tail, but not to the sixth transmembrane domain, because treatment with proteinase K in the absence of detergent removes all staining with the α-TIP antiserum. Only the α-TIP–derived portion (the carboxy terminus) appears to be accessible to the protease and is therefore cytoplasmic. In summary, the results of this experiment confirmed that PAT-TIP is in a type I membrane protein orientation.

A Transient Expression System to Study Transport to the Vacuole

The above data demonstrate that stably transformed plants and suspension cultured cells can be used to study the transport of a protein to the tonoplast. The amount of time required to develop transgenic plants or cell lines, however, is a major limitation of those systems. For this reason, we investigated whether we could use a transient expression system to study transport to the tonoplast. Isolated tobacco leaf protoplasts were transfected with DNA, using a polyethylene glycol–based transformation procedure. Next, the cells were allowed to recover

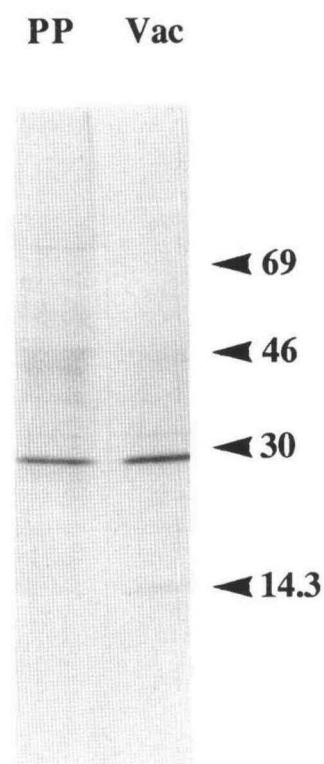

Figure 7. Localization of PAT-TIP in the Vacuoles of Transgenic Tobacco Cells.

Fluorograph of PAT-TIP immunoprecipitated from extracts from protoplast (PP) and vacuoles (Vac) containing the same amount of activity of the vacuolar marker enzyme acid phosphatase. Tobacco protoplasts were labeled with ^{35}S-labeled amino acids for 1.5 hr and chased for 5 hr prior to the vacuole preparation. Molecular size markers at right are as given in Figure 1.

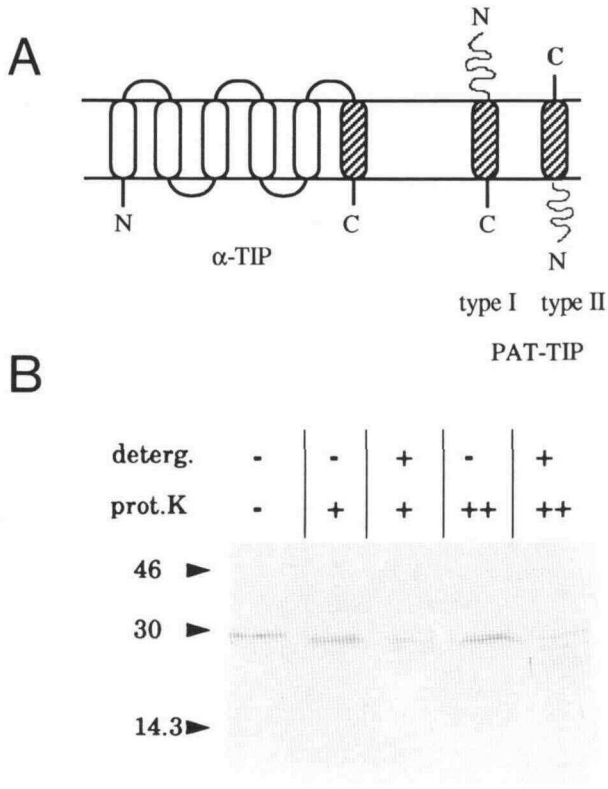

Figure 8. Orientation of PAT-TIP in the Membrane.

(A) Schematic representation of the predicted orientation of α-TIP (left) and of the possible orientations that the fusion PAT-TIP could adopt in the membrane (right). The hatched area refers to the sixth predicted transmembrane domain of α-TIP.

(B) Immunoblot with an anti-PAT antiserum on proteins extracted from vacuoles isolated from transgenic tobacco cells after treatment with 0, 100 μg (+), or 300 (++) μg proteinase K (prot. K) in the presence or absence of detergent (0.75% Chaps). Note that proteinase K slightly increases the mobility of the cross-reacting protein in the absence of detergent, confirming the type I orientation of the protein. Size markers at left are given in kilodaltons.

for 4 hr, then labeled for 1 hr, and chased for increasing time periods; vacuoles were then isolated and analyzed using the techniques described above. Figure 9A shows that α-TIP could be detected in transiently transfected protoplasts (lane 1). In addition, some radioactive α-TIP was already present in the purified vacuoles after a 1-hr pulse, and most if not all of the labeled α-TIP protein in the protoplast reached the vacuole after a 5-hr chase. Immunoprecipitations with anti-BiP antibodies again confirmed that the contamination of the vacuoles with ER membranes was limited (Figure 9B). In conclusion, the transport of α-TIP seems to follow similar kinetics both in this transient expression system and in the stably transformed cells, indicating that this transient system can be used for the study of the targeting of tonoplast proteins.

TIPΔ15, a Deletion Mutant of α-TIP Lacking the Cytoplasmic Tail, Is Still Targeted to the Tonoplast

The results presented so far support the idea that the carboxy-terminal 48 amino acids of α-TIP containing the sixth transmembrane domain and the cytoplasmic tail are sufficient to redirect a secreted protein to the tonoplast. To investigate whether the same portion of α-TIP is also necessary for tonoplast targeting, we constructed a deletion derivative of α-TIP lacking exactly the 48 carboxy-terminal amino acids. Unfortunately, the extreme instability of this protein (data not shown) prevented us from studying its intracellular localization. A second deletion derivative, TIPΔ15, in which the carboxy-terminal 15 amino acids were deleted, was used to study the requirement of the cytoplasmic carboxy terminus for the targeting of α-TIP to the tonoplast. DNA constructs containing either the α-TIP gene or the α-TIPΔ15 mutant were

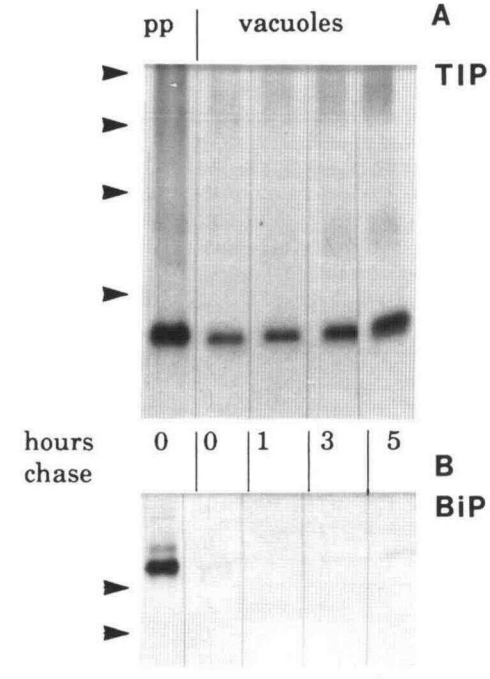

Figure 9. Accumulation in Vacuoles of α-TIP Transiently Expressed in Tobacco Leaf Protoplasts.

(A) Fluorograph of α-TIP.

(B) Fluorograph of BiP.

α-TIP and BiP were immunoprecipitated from vacuolar extracts (last four lanes) and a protoplast (pp) extract (first lane) containing the same activity of the vacuolar marker enzyme acid phosphatase. Protoplasts were labeled for 1 hr, and the radioactivity was chased respectively for 0, 1, 3, and 5 hr.

Note that the amount of α-TIP in the vacuolar fraction increases with time (arrowheads in **[A]** are the same as in Figure 1). Little or no BiP is detectable in the vacuolar fraction, indicating that the vacuolar preparation is not contaminated with ER membranes (arrowheads in **[B]** are molecular size markers of 69 and 43 kD).

transiently expressed in leaf protoplasts, vacuoles were isolated after 4.5-hr labeling, followed by a 3.5-hr chase, and the proteins were immunoprecipitated with antibodies to α-TIP, as shown in Figure 10B. Immunoprecipitations with anti-BiP antibodies served as a control for the purity of the vacuole preparations (Figure 10A). First, the absence of the cytoplasmic tail seemed to interfere substantially with the stability of the TIPΔ15 protein (data not shown). Second, despite the absence of the cytoplasmic tail, the TIPΔ15 protein also could be detected in the vacuole fraction (Figure 10B, fourth lane). The weaker signal in the vacuole fraction after 3.5 hr of chase compared to the protoplast fraction could indicate incomplete arrival in the tonoplast after this brief chase. We were not able to detect the protein after longer chase periods, probably due to the instability of the protein. These data indicate that the 15 amino acids constituting the cytoplasmic tail are not necessary for the targeting of α-TIP to the tonoplast.

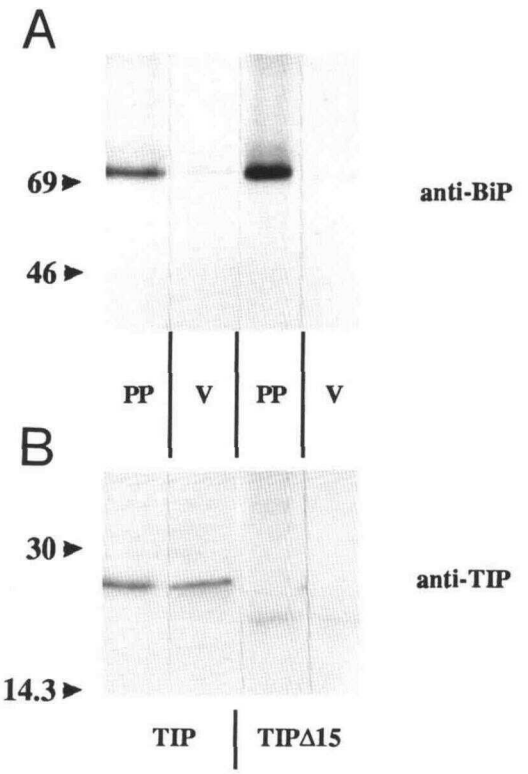

Figure 10. Vacuolar Localization of TIPΔ15, a Deletion Mutant of α-TIP Lacking the Cytoplasmic Tail, Transiently Expressed in Tobacco Leaf Protoplasts.

(A) Fluorograph of BiP.
(B) Fluorograph of TIP.
The proteins were immunoprecipitated from protoplast (PP) and vacuolar (V) extracts containing the same amount of activity of the vacuolar marker enzyme acid phosphatase. Antisera were raised against α-TIP **(B)** or the ER resident protein BiP **(A)**. Tobacco protoplasts were transfected with pTIP14 (P35S-α-TIP-3′ octopine synthase [ocs] gene, first two lanes marked as TIP) or pTIP14Δ15 (P35S-α-TIPΔ15-3′ ocs, last two lanes marked as TIPΔ15), were labeled for 4.5 hr, and the radioactivity was chased for 3.5 hr prior to the isolation of vacuoles. Molecular size markers at left are given in kilodaltons.

DISCUSSION

This report shows that a polypeptide segment with one transmembrane domain of α-TIP is sufficient to target a passenger protein to the tonoplast. We previously showed that α-TIP, a seed protein in the membrane of protein storage vacuoles, is correctly targeted to the tonoplast in leaves of transgenic tobacco. This result indicates that the cellular machinery involved in the targeting of membrane proteins to the tonoplast is conserved in different tissues (Höfte et al., 1991). Interestingly, the transport rate for α-TIP to the tonoplast was significantly higher in leaf protoplasts ($t_{1/2}$ of 2 to 3 hr) than in bean cotyledons ($t_{1/2}$ of 6 to 8 hr; Mäder and Chrispeels, 1984). This difference in transport rates could reflect differences in the physiology of leaf protoplasts compared to storage parenchyma cells in developing bean seeds. Altabella et al. (1990) and Hager et al. (1991) observed that auxin stimulates the vesicular transport of another membrane protein (H^+-ATPase) to the plasma membrane. In our transport experiments, we also used a high concentration of auxin in the protoplast culture medium. It will be interesting to see whether the transport rate of α-TIP, other membrane proteins, and perhaps also soluble proteins in the secretory system is influenced by the auxin concentration and perhaps by other physiological conditions.

The cytosolic protein PAT from *S. hygroscopicus* fused to a signal peptide (from PR-1 from potato) was secreted from suspension cultured cells, confirming previous observations on leaf protoplasts (Denecke et al., 1990). The same authors also observed that the steady state accumulation level of three heterologous cytosolic reporter proteins in plant cells was significantly higher than that of the same proteins fused to a signal peptide. The pulse-chase experiment described in this report provides a possible explanation for this phenomenon. Indeed, only a small proportion of the PAT protein synthesized and imported into the rough ER (all PAT protein was imported in the ER because no ssPAT precursor could be detected at the beginning of the chase) appeared in the culture medium even after all the labeled protein had disappeared from the cell (Figure 3). This finding and the observation that PAT is stable in the culture medium (Denecke et al., 1990; H. Höfte, unpublished data) suggest that a significant portion of the PAT protein is degraded in an intracellular compartment of the secretory system. It is unlikely that this compartment is the vacuole because PAT was stable when targeted as a fusion protein to this organelle.

To facilitate the study of the transport of vacuolar and tonoplast proteins, we developed a transient assay system. The following observations support the reliability of this assay. First, the transport kinetics of α-TIP in this transient assay

were comparable to those in stably transformed cells. Second, little if any of the resident ER protein BiP could be detected in the vacuolar fraction, suggesting that the vacuoles were free of ER and that the polyethylene glycol treatment did not induce the fusion of intracellular membranes. Third, the soluble vacuolar protein phytohemagglutinin, transiently expressed in these protoplasts, also accumulated in the vacuoles in a time-dependent fashion (data not shown). In addition, phytohemagglutinin was transported via the Golgi apparatus because the typical, time-dependent shifts in mobility could be observed on fluorographs, consistent with the modifications of the glycan side chains (Vitale et al., 1984). The reliability of the assay depends on the purity of the vacuolar preparation. The preparation contained no BiP, a marker for the ER, but we cannot exclude the possibility that it contained no plasma membrane contaminants.

The experiments shown here demonstrate that the carboxy-terminal 48 amino acids of α-TIP contain sufficient information to transport a reporter protein to the tonoplast. Is this portion of α-TIP also necessary for tonoplast sorting? The truncated protein α-TIPΔ15, lacking the cytoplasmic tail, still accumulated in the tonoplast, eliminating the possibility that the cytoplasmic tail alone contains positive sorting information, as was observed for a number of lysosomal proteins in animal cells (Hopkins, 1992). This leaves us with the following possibilities. First, the targeting information may be contained within the predicted sixth transmembrane domain. Targeting information contained within a transmembrane domain has been demonstrated for Golgi-localized proteins in animal cells (Munro, 1991; Nilsson et al., 1991). Alternatively, but less likely, the targeting information may be redundant; i.e., the sixth transmembrane domain contains targeting signal, but other parts of α-TIP also contain targeting information. Finally, the results do not exclude the possibility that, in contrast to animal cells in which the plasma membrane is the default destination of membrane proteins, in plant cells membrane proteins may be sorted to the vacuole by a bulk flow process. The idea that the tonoplast may be a default destination in plant cells is reinforced by recent findings that in the yeast *Saccharomyces cerevisiae* transport to the vacuolar membrane occurs by default (S. F. Nothwehr, C. J. Roberts, and T. H. Stevens, personal communication). If the tonoplast is also a default destination in plant cells, positive sorting signals should be found in integral plasma membrane proteins.

METHODS

Strains, Culture Conditions, and Preparation of Protoplasts

Nicotiana tabacum suspension culture cells (tobacco cells) were kindly provided by Dr. N. Raikhel (Michigan State University, East Lansing). Culture conditions were as described by Hunt and Chrispeels (1991). Protoplasts were prepared from tobacco cells as described by Bednarek and Raikhel (1991). In vitro culture conditions of tobacco plants (*N. tabacum* cv Xanthi) and preparation of leaf protoplasts were described in Höfte et al. (1991).

Genetic Constructs

pTIP10, the vector used to express α-tonoplast intrinsic protein (α-TIP) in transgenic tobacco, was described previously (Höfte et al., 1991). pTIP14, a derivative of pTIP10 used to express α-TIP transiently in protoplasts, contains the following elements cloned between the EcoRI and the HindIII sites of the polylinker in pBS− (Stratagene): an EcoRI-NcoI fragment from pDE100 (Denecke et al., 1990) containing the 35S promoter of cauliflower mosaic virus (CaMV), the α-TIP gene from base pairs 186 to 1208 (Johnson et al., 1990), and the 3′ end of the octopine synthase gene (De Block et al., 1987). pTIP14Δ15 was constructed using the polymerase chain reaction from pTIP14. The plasmid is identical to pTIP14 except that it contains a deletion derivative of the α-TIP gene lacking base pairs 905 to 951. pDE314 (Denecke et al., 1990) was used to express signal sequence–phosphinotricine acetyltransferase (ssPAT) in transgenic tobacco cells. pTIPbar3, used to express ssPAT-TIP in tobacco cells, is pBS− and contains a fragment from pDE314 with the CaMV 35S promoter followed by the entire coding sequence of the bar gene (which encodes PAT) and α-TIP from base pairs 802 to 1208, covering the 48 carboxy-terminal codons and the 3′ untranslated sequence, followed by the 3′ end of the octopine synthase gene. To raise antisera against PAT and tobacco binding protein (BiP), the following *Escherichia coli* expression construct was made: pGST-BiP, which is a fusion between glutathione-*S*-transferase (GST) (Smith and Johnson, 1988) and 129 internal amino acids of tobacco BiP (Denecke et al., 1990). The corresponding DNA fragment was kindly provided by Dr. J. Denecke (University of Uppsala, Sweden). pGST-PAT contains the entire coding sequence of PAT (Thompson et al., 1987) fused to GST. Fusion proteins were expressed in *E. coli* and purified on a glutathione-Sepharose (Sigma) column as described by Smith and Johnson (1988). To generate transgenic tobacco cells, EcoRI-HindIII fragments containing the chimeric genes were isolated from pDE314, pTIPbar3, and pTIP14, and these were cloned into the binary plant vector pDE1001 (Denecke et al., 1990). *Agrobacterium tumefaciens* LBA4404 was transformed with the respective resulting plasmids as described by Höfgen and Willmitzer (1988) and used to transform tobacco cells according to Hunt and Chrispeels (1991).

Labeling and Extraction of Protoplasts

Freshly prepared tobacco protoplasts were incubated at a density of 10^6 protoplasts per 3 mL of MS2 medium (Hunt and Chrispeels, 1991) supplied with 0.45 M mannitol. After a 2- to 3-hr incubation at 27°C, the cells were labeled with a mixture of ^{35}S-labeled methionine and ^{35}S-cysteine (100 to 200 μCi EXPRESS 35 [Du Pont] per experimental point) for 1 hr unless stated otherwise. For the chase, an excess of nonradioactive cysteine and methionine (2.5 and 5 mM final concentration, respectively) was added. For the detection of protein in the culture medium, the cells were labeled and chased in the presence of 150 μg/mL BSA.

For protein extraction, protoplasts were vortexed in the presence of siliconized glass beads in extraction buffer (200 μL/10^6 protoplasts of 0.5 M betaine, 1 mM DTT, 1 mM EDTA, 50 mM Hepes, pH 8, 0.5 mM phenylmethylsulfonyl fluoride [PMSF], 0.1 μg/mL leupeptin, 10^{-7} M pepstatin). Cell debris was removed by centrifugation at 100*g* for

5 min at 4°C. The supernatant was used for immunoprecipitation, immunoblotting, or enzyme assays.

Immunodetection

Immunoblots were carried out with horseradish peroxidase–coupled secondary antibodies according to the specifications of the manufacturer (Bio-Rad). A rabbit antiserum raised against bean α-TIP was described previously (Johnson et al., 1990). Fusion proteins between GST, and PAT and BiP, respectively, were used to raise rabbit antisera. For immunoprecipitations, proteins were first precipitated with 7% trichloroacetic acid (TCA) and centrifuged, and the pellet was washed with 70% acetone and resuspended in 180 μL of Tris-buffered saline (TBS). A brief pulse with the sonicator facilitated resuspension of the pellet if needed. Twenty microliters of 10% SDS was added, and the sample was boiled for 5 min (samples with PAT or PAT-TIP) or heated at 65°C for 10 min (for samples with α-TIP, because boiling caused the polypeptides to aggregate). After the addition of 800 μL TBS + 1.25% Triton X-100, the sample was heated for 2 min at 65°C and centrifuged for 15 min in an Eppendorf centrifuge. Antiserum (5 μL) was added, and the mixture was incubated on ice for 1 hr. Preswollen protein A–Sepharose (10 mg in 100 μL) was added to the supernatants, and the sample was tumbled at 4°C for 1 hr. Beads were collected through centrifugation for 1 min, and were washed three times with TBS + 0.1% SDS + 1% Triton X-100, three times with TBS + 1% Triton X-100, and three times with TBS. After the last wash, the beads were resuspended in 20 μL of SDS-PAGE sample buffer and treated for 10 min at 65°C (or 1 min at 100°C for samples with PAT or PAT-TIP); the supernatant was analyzed on 15% SDS–polyacrylamide gels followed by fluorography.

Vacuole Preparations

Vacuoles were prepared from leaf protoplasts essentially as described previously (Höfte et al., 1991) but with 0.5% BSA in the lysis buffer and with 0.6 M betaine, instead of 0.5 M mannitol as osmoticum used to prepare the two upper layers of the gradient. In addition, the vacuole buffer was supplemented with 150 μg/mL BSA, 0.5 mM PMSF, 0.1 μg/mL leupeptin, and 10^{-7} M pepstatin. Vacuoles were prepared from tobacco cells using a protocol adapted from Boudet et al. (1981). Protoplasts (3×10^6) were pelleted and resuspended in 2.5 mL of solution I (10% Ficoll, 0.45 M mannitol, and 25 mM Mes, pH 6.5) and transferred to a small glass tube and overlayered with the following solutions in the order given: 2.5 mL of solution II (6% Ficoll, 4 mg/mL DEAE dextran, 25 mM Mes, pH 6.5, 0.5 M mannitol, and 40 μg/mL DNase), 3 mL of solution III (4% Ficoll, 1.5 mg/mL dextran sulfate, 25 mM Hepes, pH 8, 0.5 M mannitol, 40 μg/mL DNase), and 0.5 mL of solution IV (0.5 M betaine, 25 mM Hepes, pH 8, 150 μg/mL BSA, 0.5 mM PMSF, 0.1 μg/mL leupeptin, 10^{-7} M pepstatin). The gradient was centrifuged for 20 min at 3000 rpm in a Sorvall centrifuge at 15°C. Vacuoles accumulated at the interphase between solutions III and IV and were collected with a Pasteur pipette.

Vacuoles were quantitated based on the activity of the marker enzyme acid phosphatase. For the enzyme assay, 100 μL of protein extracts were incubated with 300 μL of 100 mM sodium acetate, pH 5, 150 μg/mL BSA with 100 μL *p*-nitrophenyl phosphate (1 mg/mL) at room temperature. Samples of 100 μL were transferred to a microtiter plate at different time points, and the reaction was terminated with 100 μL of 1 M Na_2CO_3, pH 11. The reaction product was quantitated by reading the absorbance at 410 nm with a microtiter plate reader.

Fractionation of Microsomal Proteins

Radioactively labeled protoplasts were lysed as described above. Cell debris were removed through centrifugation at 100*g* for 5 min. The supernatant was adjusted to 100 mM Na_2CO_3, pH 11.5, and vigorously vortexed. Membranes were pelleted from the supernatant through centrifugation for 90 min at 40,000 rpm in a 50 Ti rotor (Beckman Instruments). The pellet was resuspended and vigorously vortexed in 100 mM Na_2CO_3, pH 11.5, and centrifuged again. The pellet was resuspended in extraction buffer, the two supernatants were combined, and both samples were TCA precipitated, resuspended in extraction buffer, and immunoprecipitated.

Transient Expression Assays

Tobacco leaf protoplasts were prepared from plants grown in sterile Magenta boxes, essentially as described by Höfte et al. (1991). Protoplasts were suspended in 0.5 M sucrose, 0.1% Mes, pH 5.6, and centrifuged at 60*g*; the cells floating on top of the medium were carefully collected with a Pasteur pipette and transferred to W5 medium (145 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM glucose, pH 5.5 to 6). The use of 15-mL ampules with long, slender necks facilitated the collection of the protoplasts, which accumulated in the neck of the ampule. The transient expression procedure was adapted from Negrutiu et al. (1987). Protoplasts were counted in a hemocytometer, incubated on ice for 30 min, and pelleted as described above. The pellet was resuspended in 0.5 M mannitol, 15 mM $MgCl_2$, 0.1% Mes-KOH, pH 5.6, to a density of 1.6×10^6 per mL. DNA (15 μg) was placed in sterile glass tubes, and 300 μL of protoplast suspension was added and gently mixed with an automatic pipette tip from which the end had been cut off. Next, 500 μL of PEG solution (40*g* PEG 8000 added to 100 mL of 0.4 M mannitol, 0.1 M calcium nitrate, 0.1% Mes-KOH, pH 7) was added in a dropwise fashion. After a 30-min incubation at room temperature, the PEG solution was diluted by the addition of 5×1 mL W5 medium in 5-min intervals; the tube was swirled after each addition. The protoplasts were pelleted as above and resuspended in B5t (complete Gamborg medium from Sigma) supplied with 0.5 M glucose adjusted to pH 5.6 with KOH and 1 mg/L naphthaleneacetic acid, 0.2 mg/L benzylaminopurine, 0.1 mg/L 2,4-D at a final concentration of 10^6 protoplasts in 3 mL of medium. Protoplasts were incubated for at least 4 hr at 27°C in the dark before labeling with EXPRESS 35 and chase (see above).

Protease Protection Assay

Vacuoles were prepared from 8×10^6 tobacco protoplasts as described above, except that no BSA or protease inhibitors were added to buffer IV, and aliquots of vacuoles representing the yield from approximately 1.3×10^6 protoplasts were treated with 100 or 300 μg proteinase K in 400 μL 0.6 M betaine, 10 mM $CaCl_2$, 50 mM Tris/HCl, pH 7.5 in the presence or absence of 0.75% Chaps detergent for 60 min on ice. Proteins were TCA precipitated and centrifuged; the pellets were washed with 70% acetone, dissolved in sample buffer, and analyzed on an immunoblot with anti-PAT antibodies.

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