

RESEARCH ARTICLE

Tonoplast Intrinsic Protein Isoforms as Markers for Vacuolar Functions

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Plant cell vacuoles may have storage or lytic functions, but biochemical markers specific for the tonoplasts of functionally distinct vacuoles are poorly defined. Here, we use anti-peptide antibodies specific for the tonoplast intrinsic proteins α -TIP, γ -TIP, and δ -TIP in confocal immunofluorescence experiments to test the hypothesis that different TIP isoforms may define different vacuole functions. Organelles labeled with these antibodies were also labeled with anti-pyrophosphatase antibodies, demonstrating that regardless of their size, they had the expected characteristics of vacuoles. Our results demonstrate that the storage vacuole tonoplast contains δ -TIP, protein storage vacuoles containing seed-type storage proteins are marked by α - and δ - or α - and δ - plus γ -TIP, whereas vacuoles storing vegetative storage proteins and pigments are marked by δ -TIP alone or δ - plus γ -TIP. In contrast, those marked by γ -TIP alone have characteristics of lytic vacuoles, and results from other researchers indicate that α -TIP alone is a marker for autophagic vacuoles. In root tips, relatively undifferentiated cells that contain vacuoles labeled separately for each of the three TIPs have been identified. These results argue that plant cells have the ability to generate and maintain three separate vacuole organelles, with each being marked by a different TIP, and that the functional diversity of the vacuolar system may be generated from different combinations of the three basic types.

INTRODUCTION

Plant vacuoles have multiple functions (Wink, 1993). They may contain hydrolytic enzymes and function as a digestive organelle similar to lysosomes in animal cells (Boller and Kende, 1979). They may contain secondary metabolic products such as alkaloids, glycosides and glutathione conjugates, organic acids, and anthocyanidins (Wink, 1993). They may also store proteins in seed (Müntz, 1998) and vegetative tissues (Staswick, 1994). Although previous models hypothesized that these diverse functions occurred within a single vacuole, it is now clear that protein storage vacuoles (PSVs), containing seed-type storage proteins, and lytic vacuoles, marked by the presence of active proteases, are separate organelles (Hoh et al., 1995; Paris et al., 1996). Recently, a third functionally distinct type of vacuole that stores pigments and vegetative storage proteins synthesized in response to developmental and environmental cues in various plant tissues was defined (Jauh et al., 1998).

These studies have demonstrated an apparent association between tonoplast intrinsic proteins (TIPs) and vacuolar function. TIPs are integral membrane proteins in the tonoplast

(Johnson et al., 1989) and represent a distinct group within the ubiquitous membrane intrinsic protein family (Maurel, 1997). It has been suggested that TIPs function as aquaporins to regulate water transport in various vacuolar functions (Chrispeels and Maurel, 1994; Maurel, 1997). However, TIPs are very abundant proteins in the tonoplast. For example, in radish taproot, γ -TIP comprises 30 to 50% of the total tonoplast protein, whereas vacuolar pyrophosphatase and H⁺-ATPase account for only ~10% (Higuchi et al., 1998). This large amount of γ -TIP would seem to be in excess of that needed merely for physiological water transport and suggests an additional structural function for the protein (Higuchi et al., 1998).

We have proposed that the presence of a specific TIP isoform may define the function of the vacuole (Jauh et al., 1998; Neuhaus and Rogers, 1998). This idea is supported by evidence that different types of vacuoles have distinct TIP isoforms. Using confocal immunofluorescence, Paris et al. (1996) identified two functionally distinct types of vacuoles in pea and barley root tip cells. PSVs had α -TIP in their tonoplast and contained barley lectin. Lytic vacuoles were identified using the TIP-Ma27 antiserum (Marty-Mazars et al., 1995) and contained aleurain, a barley cysteine protease (Holwerda et al., 1990). Hoh et al. (1995) demonstrated that

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anti- α -TIP and TIP-Ma27 antisera labeled separate vacuoles in developing pea cotyledons. Both of these studies, however, were complicated by uncertainty over the specificity of the TIP-Ma27 antibodies. Although initially suggested to have specificity to γ -TIP (Marty-Mazars et al., 1995), the antibodies were raised by immunization of a rabbit with whole beet root tonoplast membranes, and recent results with *Arabidopsis* indicate that they predominantly recognize proteins of ≥ 45 kD rather than the expected 26 kD for a TIP (da Silva Conceição et al., 1997).

It was therefore essential to develop antibody reagents that would identify individual, specific TIP isoforms so that the tonoplast composition of lytic vacuoles could be determined and compared with that of other vacuole types. By comparing the amino acid sequences of TIP isoforms, we found that the C-terminal cytoplasmic tail sequences were diverse enough to allow generation of antibodies specific for the different TIPs. We had prepared antipeptide antibodies specific to the C-terminal amino acid sequences of α -, γ -, and δ -TIP and dark-induced protein (DIP) (Culianez-Macia and Martin, 1993; Jauh et al., 1998). Here, we show that the anti- α -TIP and anti- γ -TIP peptide antibodies have specificities when using protein gel blot analysis and immunofluorescence labeling that are indistinguishable from antibodies raised against purified α -TIP and γ -TIP proteins. A monoclonal antibody, MAb351, prepared against the γ -TIP peptide, and the rabbit polyclonal anti- γ -TIP peptide antibodies gave identical results using the two assays.

The availability of MAb351 allowed immunofluorescence triple-labeling studies to determine the patterns of association of the different TIP isoforms and to establish their relationship to vacuoles containing barley lectin, aleurain, and arabinogalactan proteins. Our results are consistent with both an association of specific TIP isoforms with specific vacuole functions and patterns of expression of TIP isoforms that reflect stages of cell development or differentiation.

RESULTS

Characterization of Anti-TIP Peptide Antibodies

The specificity of each affinity-purified anti-TIP peptide antibody was documented by using dot blot analyses with different synthetic TIP peptides coupled with BSA and protein gel blot analyses with different plant tissue extracts (Figure 1). As shown in Figure 1A, each anti-TIP peptide antibody showed at least 100-fold higher affinity for its corresponding peptide than for any of the other peptides. Similar results were obtained for the monoclonal antibody to the γ -TIP peptide, MAb351 (data not shown). Our previous results indicated that the antipeptide antibodies for α -TIP and γ -TIP gave results that were indistinguishable from results obtained with antisera raised against the entire protein se-

quences (Jauh et al., 1998). The specificity of MAb351 was also examined by using protein gel blot analysis to compare anti- α -TIP and anti- δ -TIP peptide antibodies (Figure 1B). The anti- α -TIP antibodies identified a major band of the appropriate 26-kD size, but only in the pea root tip (Figure 1B, lane 1) and pea seed (lane 5) extracts. MAb351 recognized appropriate bands of γ -TIP in pea root tip (Figure 1B, lane 6), radish (lane 7), and soybean leaf (lane 9) extracts. These results were indistinguishable from those obtained with the rabbit polyclonal anti- γ -TIP peptide antibodies (Jauh et al., 1998). However, no γ -TIP antigen was detectable in the pea seed extract (Figure 1B, lane 10). Therefore, the ~ 26 -kD band detected by MAb351 in the pea root tip extract is γ -TIP and does not represent cross-reactivity with α -TIP. The anti- δ -TIP peptide antibodies identified appropriate bands not only in pea root tip (Figure 1B, lane 11) and petunia petal (lane 13) extracts but also in the pea seed extract (lane 15). Because the soybean leaf came from a plant with pods (Jauh et al., 1998), only a small amount of δ -TIP present was detected as a dimer. In barley root tip extracts, these three anti-TIP peptide antibodies gave results similar to those obtained with pea root tip extracts (data not shown).

These results together demonstrate a high degree of specificity of each anti-TIP peptide antibody preparation to its corresponding protein. The additional bands of ~ 40 kD (Figure 1B) represent TIP dimers that form as tissue extracts are heated during preparation of samples for electrophoresis (Johnson et al., 1989; Maeshima, 1992). In addition, the presence of smaller forms of TIP monomers, compared with the intact size of ~ 26 kD (Figure 1B), is due to proteolytic cleavage of the first transmembrane domain from the rest of the protein in certain plant tissues (Inoue et al., 1995).

Because the anti- α -TIP and anti- δ -TIP antibodies frequently were observed to label the same organelles (see below), we performed an additional control experiment to demonstrate their specificity, as shown in Figure 1C. Protein gel blot strips of pea root tip extracts were incubated with the anti- α -TIP peptide antibodies in the absence of synthetic peptide (Figure 1C, lane 2) or in the presence of a vast excess of the δ -TIP peptide (lane 3) or the α -TIP peptide (lane 1). The α -TIP peptide completely prevented recognition by the antibodies of ~ 26 -kD α -TIP, whereas the δ -TIP peptide had no effect. Similarly, protein gel blots using extracts from petunia flower petals (Figure 1C, lanes 4 to 6) were incubated with anti- δ -TIP peptide antibodies. These antibodies recognized bands representing monomeric and dimeric (Figure 1C) forms of δ -TIP in the absence of added peptide (lane 5), and that pattern was not affected by the presence of a vast excess of α -TIP peptide (lane 4). In contrast, the δ -TIP peptide completely abolished recognition by the antibodies (Figure 1C, lane 6). This competition assay was repeated on samples prepared from pea seeds, in which both α - and δ -TIP are present together (Figure 1D). Again, the α -TIP peptide prevented binding by the anti- α -TIP but not the anti- δ -TIP antibodies, and vice versa. We conclude that these results are consistent with those shown

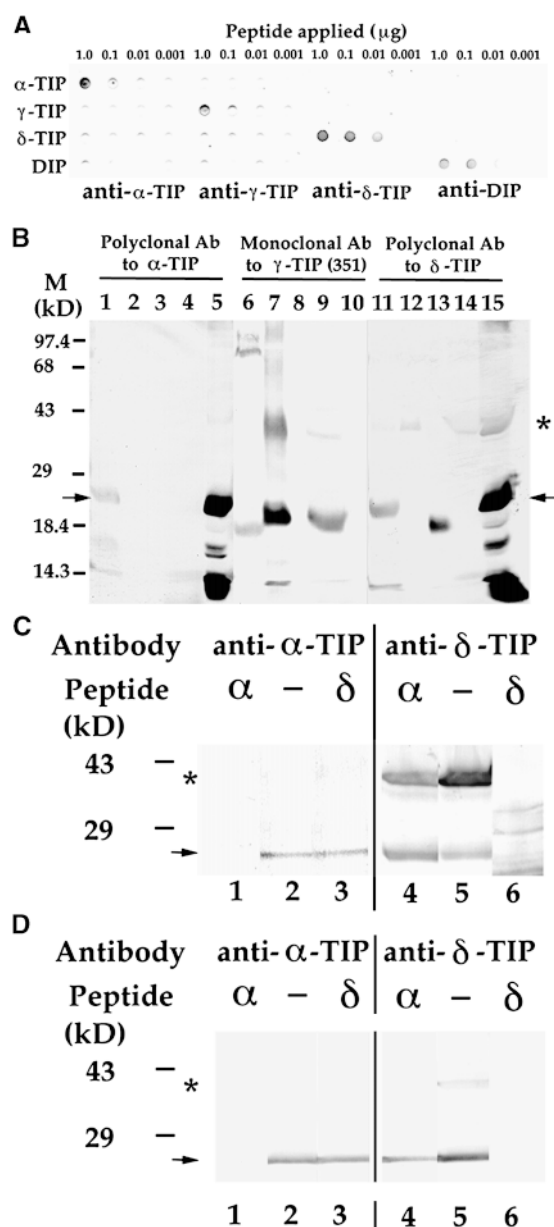


Figure 1. Characterization of the Specificity of the Antipeptide Antibodies by Immunoblot Analyses.

(A) Dot blot analyses. Four different quantities (as indicated above the blots) of each of four different BSA-conjugated synthetic peptides were applied to each of four blots. Each blot was incubated with one affinity-purified anti-TIP peptide antibody preparation used at the following concentrations: anti- α -TIP peptide antibodies, 1.2 $\mu\text{g}/\text{mL}$; anti- γ -TIP peptide antibodies, 1 $\mu\text{g}/\text{mL}$; anti- δ -TIP peptide antibodies, 1.9 $\mu\text{g}/\text{mL}$; and anti-DIP peptide antibodies, 1 $\mu\text{g}/\text{mL}$.

(B) Protein gel blots of various plant extracts probed with different anti-TIP peptide antibodies. Extracts of the following plant tissues, 300 μg per lane, were separated by SDS-PAGE and transferred to nitrocellulose membranes: pea root tips (lanes 1, 6, and 11), radish root (lanes 2, 7, and 12), petunia petals (lanes 3, 8, and 13), soybean

in Figure 1A and further emphasize the specificity of the anti- α - and anti- δ -TIP peptide antibodies.

Results from protein gel blot analyses indicate that anti-peptide and antiprotein antibodies for both γ - and α -TIP had similar specificities (Jauh et al., 1998). We determined that the two types of antibodies also gave similar results with immunofluorescence staining. Results from immunofluorescence double labeling with anti-TIP peptide antibodies and their corresponding anti-TIP antisera using pea root tip cells are shown in Figures 2A to 2C. The anti- α -TIP peptide (green) and anti- α -TIP (red) antibodies gave identical staining patterns, as demonstrated by the uniform yellow color in the merged image (Figure 2A). Similarly, the monoclonal MAb351 anti- γ -TIP peptide antibody gave a pattern indistinguishable from the anti- γ -TIP protein (Figure 2B) and the anti- γ -TIP peptide (Figure 2C) antibodies. Controls in all double-labeling experiments in which two different rabbit antibodies were used support the specificity of patterns shown: (1) When root tip cells labeled with the anti-TIP peptide antibodies were incubated with an excess of rhodamine-conjugated anti-rabbit IgG F(ab')₂ secondary antibodies, washed, and then incubated with the Cy5-conjugated anti-rabbit IgG secondary antibodies, no Cy5 labeling was obtained (data not shown). This demonstrates that the F(ab')₂ antibodies completely blocked all of the TIP antibody sites so that Cy5 labeling apparent in Figure 2 must be due to the

leaves (lanes 4, 9, and 14), and mature pea seeds (lanes 5, 10, and 15). Individual blots were incubated with anti- α -TIP peptide polyclonal antibodies (1.2 $\mu\text{g}/\text{mL}$; lanes 1 to 5), anti- γ -TIP peptide MAb351 antibody (1 $\mu\text{g}/\text{mL}$; lanes 6 to 10), and anti- δ -TIP peptide polyclonal antibodies (1.9 $\mu\text{g}/\text{mL}$; lanes 11 to 15). Arrows indicate the position of TIP monomers, and the asterisk indicates the position of TIP dimers. Ab, antibodies; M, molecular mass markers with size indicated in kilodaltons.

(C) Competition assay for anti-TIP antibodies. Extracts of pea root tips (lanes 1 to 3) and petunia petals (lanes 4 to 6), 300 μg per lane, were separated by SDS-PAGE and transferred to nitrocellulose membranes. Individual blots were incubated with anti- α -TIP peptide polyclonal antibodies (1.2 $\mu\text{g}/\text{mL}$) preincubated with 10 $\mu\text{g}/\text{mL}$ BSA-conjugated α -TIP peptides (lane 1), anti- α -TIP peptide polyclonal antibodies (1.2 $\mu\text{g}/\text{mL}$; lane 2), anti- α -TIP peptide polyclonal antibodies (1.2 $\mu\text{g}/\text{mL}$) preincubated with 10 $\mu\text{g}/\text{mL}$ BSA-conjugated δ -TIP peptides (lane 3), anti- δ -TIP peptide polyclonal antibodies (1.2 $\mu\text{g}/\text{mL}$; lane 4), anti- δ -TIP peptide polyclonal antibodies (1.2 $\mu\text{g}/\text{mL}$; lane 5), and anti- α -TIP peptide polyclonal antibodies (1.2 $\mu\text{g}/\text{mL}$) preincubated with 10 $\mu\text{g}/\text{mL}$ BSA-conjugated δ -TIP peptides (lane 6). Numbers at left are molecular mass markers with size indicated in kilodaltons; the arrow indicates the position of TIP monomers, and the asterisk indicates the position of TIP dimers.

(D) Competition assay for anti-TIP antibodies using extracts from dry pea seeds. Samples (300 μg) were electrophoresed, and the competition assay was performed as in **(C)**. Lane designations and figure labeling are also as in **(C)**.

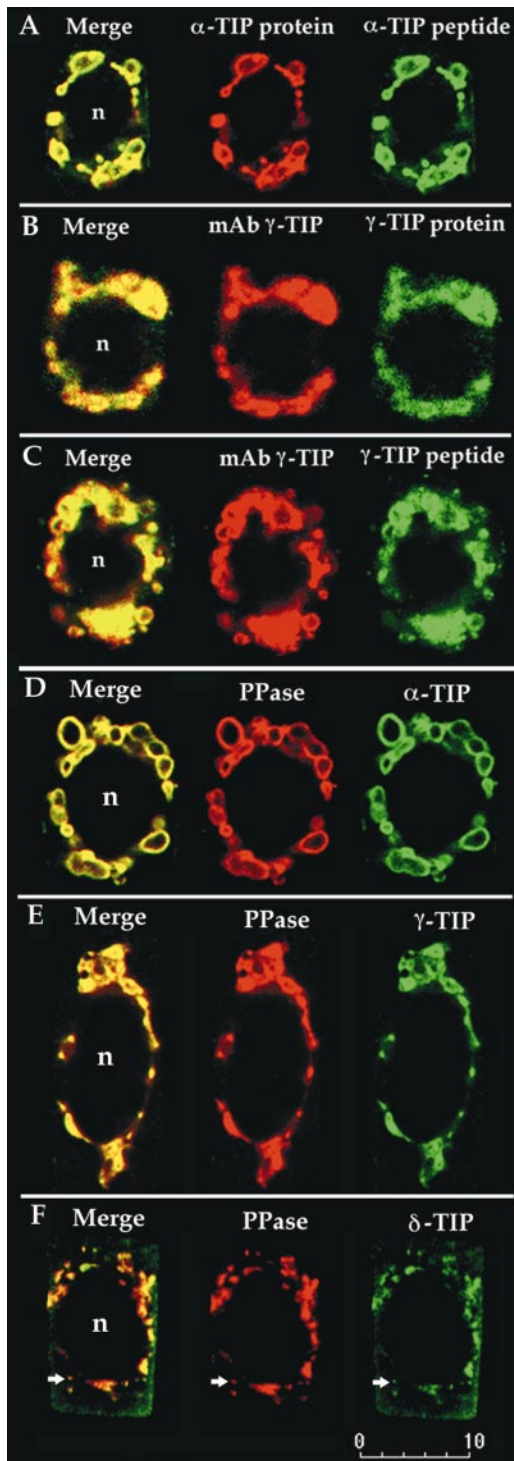


Figure 2. Colocalization of Anti-TIP Peptide Antibodies with Their Corresponding Anti-Protein Antibodies and with Antivacuolar H^+ Pyrophosphatase Antibodies in Pea Root Tip Cells.

(A) Isolated pea root tip cells were double-labeled with anti- α -TIP peptide antibodies ($1.2 \mu\text{g}/\text{mL}$) and anti- α -TIP protein antiserum (1:1000).

presence of the second anti-TIP peptide/protein antibodies at those sites. (2) The labeling patterns observed were obtained regardless of the order in which the two anti-TIP antibodies were used. These results indicate that our anti-TIP peptide antibodies recognized the same TIP proteins that are recognized by the corresponding anti-TIP protein antibodies. The anti-peptide antibodies were therefore used exclusively in all subsequent experiments. In addition, the MAb351 and rabbit anti- γ -TIP peptide antibodies have identical specificities. This fact made triple immunofluorescence labeling studies possible (see below).

We then compared labeling patterns of the anti-TIP peptide antibodies with patterns obtained with antibodies raised against vacuolar proton-translocating pyrophosphatase (V-PPase; Maeshima and Yoshida, 1989). As shown in Figure 2B, antibodies to the purified mung bean V-PPase (Maeshima and Yoshida, 1989) colocalized on intracellular organelles exactly with anti- α -TIP (Figure 2D), anti- γ -TIP (Figure 2E), and anti- δ -TIP (Figure 2F) peptide antibodies. Note that the anti-TIP and anti-V-PPase antibodies colocalized on organelles as small as $\sim 0.2 \mu\text{m}$ (e.g., Figure 2F, arrows). (The anti- δ -TIP antibodies additionally labeled very small punctate structures at the periphery of some cells; see below.) Similar results were obtained with antiserum 324 raised against a synthetic peptide representing hydrophilic loop IV (Sarafian et al., 1992) (data not shown). Controls as described for Figures 2A to 2C demonstrated that labeling patterns shown were dependent on the presence of both anti-TIP and anti-V-PPase antibodies and were not an artifact of the double-labeling procedure. In other experiments, there was no correlation between the labeling pattern obtained with anticalexin antibodies, a specific marker for the endoplasmic reticulum, and the anti-TIP peptide antibodies (data not shown); thus, we believe the small organelles labeled with anti-TIP antibodies are separate from the endoplasmic reticulum. Because V-PPase is "an abundant and

(B) Anti- γ -TIP MAb351 monoclonal antibody ($1 \mu\text{g}/\text{mL}$) and anti- γ -TIP protein VM23 antiserum (1:200).

(C) Anti- γ -TIP peptide polyclonal antibodies ($1 \mu\text{g}/\text{mL}$) and anti- γ -TIP peptide MAb351 monoclonal antibody ($1 \mu\text{g}/\text{mL}$).

(D) Anti- α -TIP peptide antibodies ($1.2 \mu\text{g}/\text{mL}$) and antipyrophosphatase antiserum (1:500).

(E) Anti- γ -TIP peptide antibodies ($1 \mu\text{g}/\text{mL}$) and antipyrophosphatase antiserum (1:500).

(F) Anti- δ -TIP peptide polyclonal antibodies ($0.87 \mu\text{g}/\text{mL}$) and antipyrophosphatase antiserum (1:500). The arrows indicate an $\sim 0.2 \mu\text{m}$ structure labeled with both antibodies.

Double-labeling techniques and secondary antibodies are described in Methods. For presentation purposes, the Cy-5 images were pseudocolored in green and the rhodamine images in red; colocalization of the two antibodies is indicated by yellow in the merged images. Bar in (F) = $10 \mu\text{m}$ for (A) to (F). mAb, monoclonal antibody; n, nucleus; PPase, antipyrophosphatase.

universal component of plant vacuolar membranes" (Zhen et al., 1997) and because the TIP protein family is defined by proteins present in vacuole tonoplast (Weig et al., 1997), the presence of TIP and V-PPase proteins together in the same membrane at concentrations high enough to give intense labeling in our experiments provides a biochemical definition for vacuoles.

Distribution of Different TIPs in Root Tip Cells

We wanted to learn how often vacuoles with only a single TIP occurred and, when vacuoles with more than one TIP were present, if the combinations predicted a certain function or stage of development. The function of a vacuole can be assessed from its contents (see below), but assessing stages of development is more difficult; we can only make the approximation that small cells are more likely to be meristematic and therefore early in development, whereas elongated cells with large central vacuoles are fully differentiated (Rost et al., 1988). Our method for preparing the root tips resulted in the presence of only limited numbers of fully elongated cells.

Results from a study of 142 consecutively imaged, randomly selected pea root tip cells triple-labeled with α -, γ -, and δ -TIP are summarized in Table 1, with examples of each pattern presented in Figure 3. It can be seen that relatively few cells contained vacuoles with only a single TIP, 3% for α -TIP alone, 1% for γ -TIP alone, and 2% for δ -TIP alone (Table 1). Additionally, the combination of α - and γ -TIP (Figure 3C) was very unusual, being observed only in 1% of cells. But δ -TIP was commonly found with α -TIP (Figure 3B) and γ -TIP (Figure 3D), and almost half the cells studied (Table 1) contained vacuoles that labeled with all three antibodies (Figure 3A). In general, there was no association of a certain

pattern with a distinct cell type except that the large central vacuole in fully elongated cells was predominantly or exclusively labeled with anti- γ -TIP, and small, presumably meristematic cells commonly had many small vacuoles labeled with α -TIP and δ -TIP.

When two or more TIPs were present on vacuoles in the same cell, they were colocalized in a relatively uniform manner on all or nearly all of the vacuoles present; few vacuoles in such a cell would be labeled with only one of the TIP markers. Several unusual cells, however, were identified in which separate vacuoles were labeled individually with each of the three antibodies; an example of one such cell is shown in Figure 3E. Here, small vacuoles labeled separately with anti- γ -TIP, anti- δ -TIP, and anti- α -TIP can readily be identified among other vacuoles that carry various combinations of the antibodies. This pattern was reproducible in multiple optical sections through the same cell (data not shown). The importance of this observation in development of a model for biogenesis of plant vacuoles is considered below (see Discussion).

Comparison of Labeling Patterns for Anti-TIP-Ma27, Anti- δ -TIP, and Anti- γ -TIP Antibodies

Previous studies used TIP-Ma27 antiserum to identify vacuoles that contained the cysteine protease aleurain; these were termed lytic vacuoles (Paris et al., 1996). Although the TIP-Ma27 antiserum was thought to be specific for γ -TIP (Marty-Mazars et al., 1995), it was generated by immunizing a rabbit with whole beet root tonoplast and probably contained antibodies against multiple proteins. Therefore, we compared the staining pattern obtained with TIP-Ma27 to patterns obtained with anti- δ -TIP peptide antibodies in immunoelectron microscopic studies (Figure 4).

We performed immunogold labeling of thin sections from pea root tips fixed using the high-pressure, rapid freezing process (Zhang and Staehelin, 1992). As shown in Figure 4A, TIP-Ma27 labeling was present over cell wall, plasma membrane, and small vacuoles adjacent to the plasma membrane. The specificity of this pattern is indicated by the fact that gold particles were observed over other organelles, such as mitochondria, only rarely. Interestingly, much of the vacuole labeling appeared to be over interior contents, with fewer gold particles positioned over the tonoplast. However, vacuole labeling with anti- δ -TIP (Figures 4B and 4C) antibodies was predominantly over the tonoplast. Figure 4B shows small vacuoles comparable in size with those shown in Figure 4A, to emphasize the difference in labeling patterns obtained with the two different antibodies. Figure 4C shows a larger vacuole to emphasize that specific labeling of the vacuole membrane with the anti- δ -TIP antibodies was independent of the size of the vacuoles, and the specificity of this pattern is supported by the essential absence of plasma membrane and cell wall labeling (Figures 4B and 4C). It is also important to emphasize that the size of the vacuoles shown in Figures 4A and 4B is comparable with the smallest

Table 1. Percentage of Cells with Labeled Vacuoles

Antibody Colocalization	α	$\alpha+\gamma$	$\alpha+\delta$	$\alpha+\gamma+\delta$	$\gamma+\delta$	γ	δ
TIP colocalization ^a	3	1	28	48	17	1	2
TIP colocalization with barley lectin							
Experiment I ^b α and δ	14	—	77	—	—	—	7
Experiment II ^c γ and δ	—	—	—	—	76	13	11

^a A total of 142 isolated pea root tip cells were examined by confocal laser scanning microscopy; each was scored for the presence of α -, γ -, and δ -TIP on vacuoles contained within the cell; numbers represent the percentage of total cells scored with each labeling pattern as indicated at the top.

^b A total of 43 barley root tip cells selected for positive labeling for barley lectin were studied to determine labeling patterns with α - and δ -TIP antibodies; numbers are as for TIP colocalization.

^c A total of 53 barley root tip cells selected for positive labeling for barley lectin were studied to determine labeling patterns with γ - and δ -TIP antibodies; numbers are as for TIP colocalization.

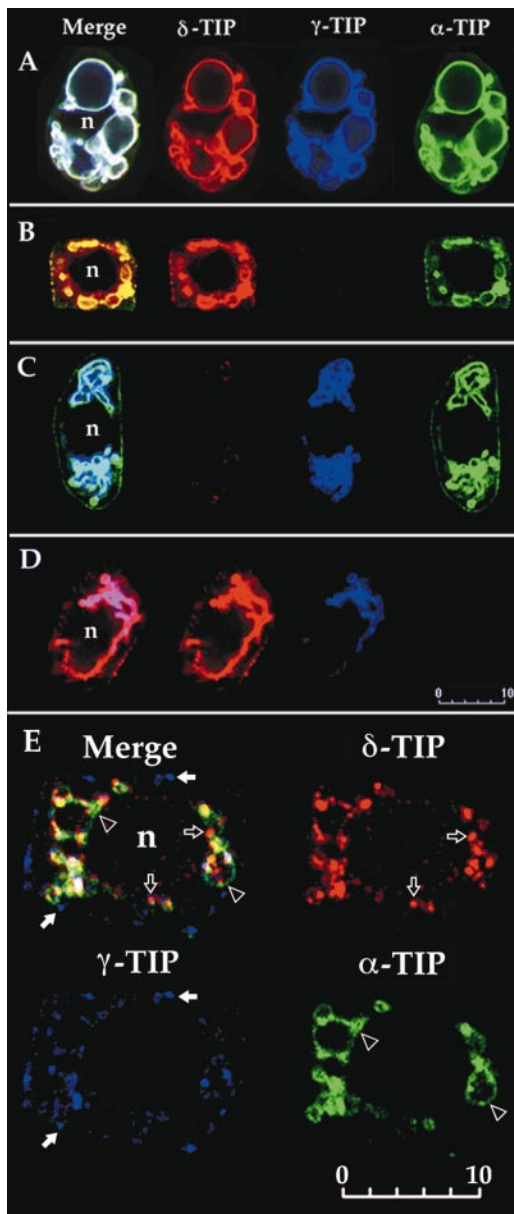


Figure 3. Immunofluorescent Triple Labeling of α -TIP, δ -TIP, and γ -TIP in Isolated Pea Root Tip Cells.

Methodology for triple labeling is described in Methods. Secondary antibodies coupled to lissamine rhodamine (for δ -TIP), Cy-5 (for γ -TIP), and FITC (for α -TIP) were used. For presentation purposes, the Cy-5 image was pseudocolored in blue, the rhodamine image in red, and the FITC image in green. Examples of each labeling pattern in which two or more TIPs were present on cell vacuoles are presented.

(A) All three TIPs are present. Colocalization of the three antibodies is indicated by the white color in the merged image (left).

(B) α - and δ -TIPs are present. Colocalization of the two antibodies is indicated by yellow in the merged image.

(C) α - and γ -TIPs are present. Colocalization of the antibodies is indicated by the aquamarine color in the merged image.

vacuoles labeled with both anti-V-PPase and anti- δ -TIP in the cell presented in Figure 2F. We conclude that these are vacuoles, not transport vesicles.

These results, together with our previous findings of strong immunofluorescence labeling at the cell periphery (Paris et al., 1996), indicate that TIP-Ma27 antiserum recognized epitopes present in cell wall, plasma membrane, and vacuole contents as well as on the tonoplast. In contrast, the anti-TIP peptide antibodies are relatively specific for tonoplast-associated epitopes.

Association of δ - and γ -TIP with Vacuoles Containing Aleurain

Previously, aleurain was found to be associated with vacuoles labeled by TIP-Ma27 (Paris et al., 1996). The results discussed above, however, mean that TIP-Ma27 labeling probably was not specific for a single TIP. Therefore, it was important to clarify exactly which TIP isoforms were associated with aleurain-containing vacuoles. As shown in Figure 5A, barley root tip cells with many small vacuoles demonstrated two predominant patterns. In one (cell 1), although both δ -TIP and γ -TIP were present together on most vacuoles (aquamarine color in merged image), aleurain was almost exclusively localized to a smaller population that carried δ -TIP alone (solid arrow). In the second pattern (cell 2), aleurain was predominantly associated with vacuoles carrying both δ -TIP and γ -TIP, although some aleurain-containing vacuoles in the same cell carried only δ -TIP. In contrast, elongated cells with large central vacuoles showed a much different pattern (Figure 5B). Presented are three consecutive optical sections through the same cell. The tonoplast was labeled with anti- γ -TIP (indicated in red). Aleurain antigen, indicated in green, was present in aggregates within the lumen of the large vacuole (examples indicated by arrows) as well as in clusters associated with the tonoplast. The clusters and aggregates presumably resulted from the fixation process. This observation emphasizes a potential cause for sampling bias when cells with aleurain-containing vacuoles were scored. Preservation of aleurain antigen in the cells requires its cross-linking to other cell proteins. This process may be much more efficient within a small volume, and thus small vacuoles may be more intensely stained and

(D) γ - and δ -TIPs are present. Colocalization of γ -TIP on a portion of the tonoplast labeled with δ -TIP is indicated by the pink color in the merged image.

(E) Cell with individual vacuoles labeled with each TIP antibody. Open arrows indicate vacuoles labeled only for δ -TIP. Solid arrows indicate vacuoles labeled only for γ -TIP. Open triangles indicate tonoplast labeled only for α -TIP.

n, nucleus. Bar in **(D)** = 10 μ m for **(A)** to **(D)**; bar in **(E)** = 10 μ m.

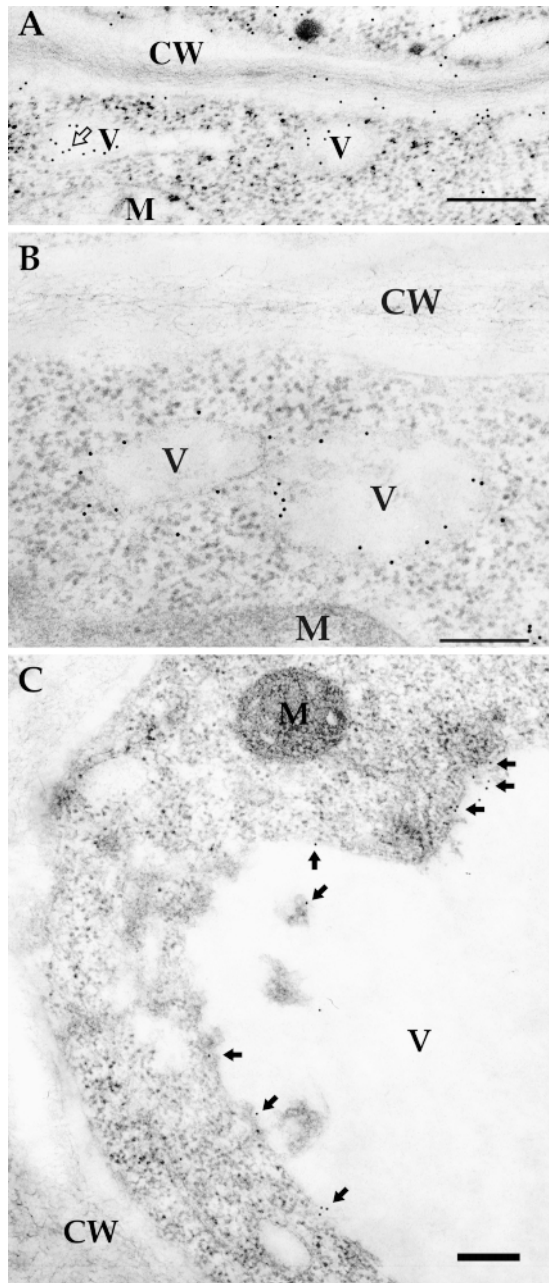


Figure 4. Electron Microscopy Immunocytochemistry with Anti-TIP-Ma27 and Anti- δ -TIP Peptide Antibodies in Pea Root Tip Sections.

(A) Labeling with anti-TIP-Ma27. The open arrow indicates gold particles from antibody labeling that are present within the lumen of the vacuole.

(B) and (C) Labeling with anti- δ -TIP antibodies. In (C), gold particles from antibody labeling are indicated with solid arrows.

CW, cell wall; M, mitochondrion; V, vacuole. Bars in (A) to (C) = 250 nm.

more readily identified when screening for the presence of aleurain is performed.

We quantified the distribution of aleurain in vacuoles carrying different TIP isoforms using such a screening procedure. As shown in Figure 6A, 93 cells from a population of barley root tip cells triple-labeled for γ -TIP, δ -TIP, and aleurain were randomly selected on the basis of a positive stain for aleurain. Of these, in five cells, aleurain was present in vacuoles that did not carry γ - or δ -TIP; in 28 cells, aleurain was present in vacuoles labeled with both γ - and δ -TIP; and in 60 cells, aleurain was present in vacuoles carrying δ -TIP but not γ -TIP. None of these cells was of the type shown in Figure 5B with a large central vacuole.

With further analysis, we noted an apparent relationship between cell morphology and labeling patterns. Vacuoles in which aleurain was present in the absence of either γ -TIP or δ -TIP were only found in larger rectangular cells, with the longest dimension being greater than two times the smallest. Cells with vacuoles labeled with aleurain plus both γ - and δ -TIP were also larger cells (e.g., Figure 5A). However, half of the cells labeled with aleurain and δ -TIP alone were the larger type, whereas the others were smaller cuboidal cells in which the longest dimension was <1.5 times the smallest, and the smallest dimension was ≤ 10 μm . These “smaller” cells with numerous small vacuoles possibly represented cells early in a developmental pathway. Their vacuoles are further characterized below.

Distribution of α -TIP on Different Vacuole Types in Root Tip Cells

Previous studies using anti- α -TIP protein antiserum (Paris et al., 1996) documented an association of α -TIP with PSVs and an association of aleurain with vacuoles lacking α -TIP. In view of the complexity of the vacuolar system that has since become apparent, we wanted to analyze large numbers of cells to clarify the association of different TIPs with vacuoles containing aleurain and with vacuoles containing barley lectin.

First, 107 cells from a population of cells double-labeled for α -TIP peptide and aleurain were randomly selected on the basis of a positive stain for aleurain and analyzed in a quantitative way by two observers for the association of aleurain with α -TIP vacuoles (Figure 6B, 0%), colocalization in occasional vacuoles ($<50\%$, meaning that aleurain was usually but not exclusively in vacuoles lacking α -TIP), colocalization in many but not all vacuoles ($\geq 50\%$), or localization of aleurain in α -TIP vacuoles in every instance (100%). As shown in Figure 6B, in 60% of the cells, there was either no colocalization of aleurain and α -TIP peptide antibodies (0%) or only occasional association of the two antigens ($<50\%$). In contrast, in 40% of the cells, most ($\geq 50\%$) or all (100%) of the aleurain-containing vacuoles were labeled for α -TIP. Interestingly, the association of aleurain with α -TIP vacuoles was

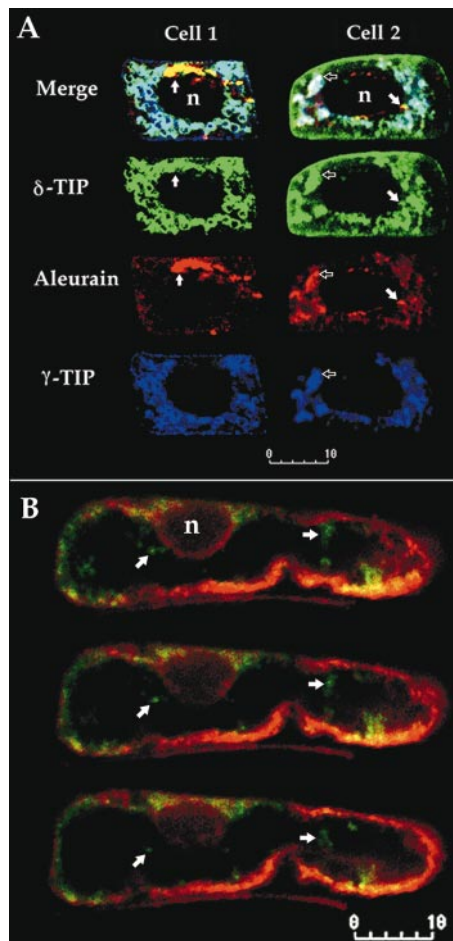


Figure 5. Aleurain in Isolated Barley Root Tip Cell Vacuoles.

(A) Aleurain in cells with small vacuoles. Immunofluorescent triple labeling of aleurain, δ -TIP, and γ -TIP: anti- δ -TIP peptide antibodies (2 μ g/mL; green), antialeurain antibodies (5 μ g/mL; red) and MAb351 anti- γ -TIP monoclonal antibody (1 μ g/mL; blue) were used. Cell 1 shows colocalization of antialeurain and anti- δ -TIP. The arrows indicate δ -TIP vacuoles that contain aleurain, as indicated by the yellow color in the merged image. These vacuoles do not label with anti- γ -TIP, whereas (as indicated by the aquamarine color in the merged image) all of the remaining δ -TIP vacuoles without aleurain also carry γ -TIP in their tonoplast. Cell 2 shows colocalization of antialeurain and both anti- δ - and anti- γ -TIP. As shown by the white color in the merged image, essentially all vacuoles containing aleurain label with both anti- δ - and anti- γ -TIP antibodies (example indicated by open arrows). One small aleurain-containing vacuole, however, is labeled by only anti- δ -TIP (solid arrows).

(B) Aleurain in an elongated cell with a large central vacuole. Serial images were collected from a root tip cell double-labeled with antialeurain (green) and anti- γ -TIP (red). Solid arrows indicate clusters of aleurain antigen within the central vacuole lumen.

n, nucleus. Bars in **(A)** and **(B)** = 10 μ m.

predominantly observed in a specific morphologic type of cells, as indicated by the designation “small” cells.

As shown in Figure 7A, left, the “small” cell type was relatively cuboidal in shape and was indistinguishable morphologically from the “smaller” cells, in which aleurain was in vacuoles marked by δ -TIP (see Figure 5A). Although we have not been able to do triple labeling to test the association directly, we hypothesize that in “small” cells, aleurain is present in vacuoles with α - and δ -TIP. In contrast, 90% of “large” cells (Figure 7A, center cell) showed no association of aleurain and α -TIP. The exception within this morphologic type was rare cells possessing what were termed “stranded” vacuoles (Figure 7A, right). These vacuoles appeared to run in a network of strands or threads for the full length of the cell and always contained both aleurain and α -TIP peptide antigens. To ensure that these results were not limited by the specificity of our anti- α -TIP peptide antibodies, we repeated the comparison of aleurain and α -TIP, using the anti- α -TIP protein antiserum (Figure 6C). In 67% of the 158 cells studied, aleurain and α -TIP were either not (0%) or only occasionally (<50%) associated, whereas in 33% they were usually (\geq 50%) or always (100%) associated, and the latter two categories were predominantly represented by small cells. Thus, the results in the two sets (Figures 7B and 7C) were similar and demonstrate that aleurain is usually not associated with vacuoles carrying α -TIP in their tonoplast, the exception being small, presumably meristematic cells.

Similar studies were performed to determine the association of α -TIP, as defined by the antipeptide antibodies, and barley lectin. When 43 cells from a population of cells triple-labeled for α -TIP, δ -TIP, and barley lectin were randomly selected on the basis of a positive stain for barley lectin, in 14% of the cells, barley lectin-containing vacuoles carried only α -TIP; in 77%, the vacuoles carried both α - and δ -TIP; and in 7%, they carried only δ -TIP (Table 1). An example is shown in Figure 8A.

In none of these cells were barley lectin-containing vacuoles found to lack both α - and δ -TIP. This observation indicates that barley lectin was not present in vacuoles carrying only γ -TIP. The association of γ -TIP with barley lectin-containing vacuoles was tested directly by screening a population of cells triple-labeled for γ -TIP, δ -TIP, and barley lectin. When 53 cells staining for barley lectin were randomly selected and analyzed, 13% carried γ -TIP alone (Table 1; this presumably represents almost all α - and γ -TIP), 11% carried δ -TIP alone (this presumably represents α - and δ -TIP), and 76% carried both γ - and δ -TIP (this presumably represents α - plus γ - plus δ -TIP). An example is shown in Figure 8B, in which arrows indicate that barley lectin is present in vacuoles labeled with both γ - and δ -TIP. Importantly, elongated cells with a large central vacuole in which the tonoplast was predominantly labeled for γ -TIP were never observed to contain barley lectin (Figure 8C).

We also conducted immunofluorescence double labeling with antialeurain and anti-barley lectin antibodies (Figure

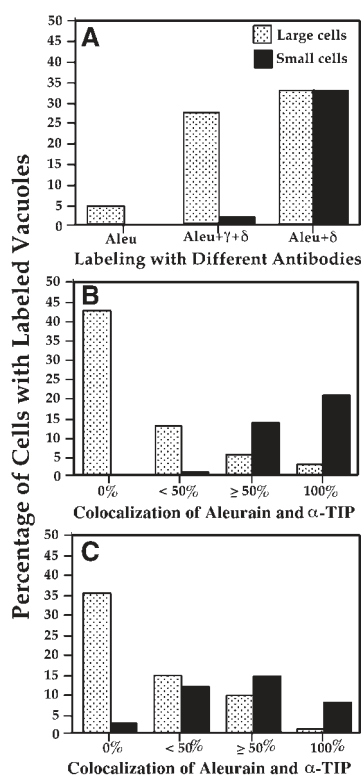


Figure 6. Quantitation of the Distribution of Aleurain in Vacuoles Carrying Different TIP Markers.

(A) Triple labeling with anti-aleurain (Aleu), anti- γ -TIP (Aleu+ γ + δ), and anti- δ -TIP (Aleu+ δ) antibodies. A total of 93 isolated barley root tip cells were identified by positive staining for aleurain, and the presence or absence of δ - and γ -TIP in the aleurain-containing vacuoles was scored. Indicated below each bar are the antibodies labeling the vacuoles of each cell; the vertical scale indicates the percentage of cells exhibiting each labeling pattern. Large cells (stippled bars) are those in which the longest dimension was $>10 \mu\text{m}$ and also greater than twice the smallest; small cells (black bars) are those in which the longest dimension was $<1.5 \times$ the shortest, and the shortest dimension was $\leq 10 \mu\text{m}$.

(B) Double labeling with anti-aleurain and anti- α -TIP peptide antibodies. A total 107 cells were identified and scored as given in **(A)**.

(C) Double labeling with anti-aleurain and anti- α -TIP protein antibodies. A total 158 cells were identified and scored as given in **(A)**. Evaluation of labeling patterns in **(B)** and **(C)** was performed by two observers as follows. Because cells were identified in which aleurain was in some vacuoles labeled with anti- α -TIP and in some vacuoles that were not labeled with anti- α -TIP, the total cells were divided into four groups, as indicated by the legend below the bar charts in **(B)** and **(C)**: 0% indicates that aleurain was not in vacuoles labeled with anti- α -TIP; 100% indicates that aleurain was exclusively in α -TIP vacuoles; $<50\%$ indicates that aleurain was predominantly but not exclusively in vacuoles that were not labeled with anti- α -TIP antibody; and $\geq 50\%$ indicates that aleurain was predominantly but not exclusively in vacuoles labeled with anti- α -TIP antibody. The scale on the ordinate and significance of light and dark bars are as given in **(A)**.

7B). When 30 barley root tip cells were examined, none of them showed colocalization of aleurain and barley lectin, suggesting that these two proteins were localized in two different cellular compartments. However, the labeling patterns appeared to be developmentally regulated. In the small cells, only aleurain was found (Figure 7B). In more elongated cells, barley lectin was found in some vacuoles (Figure 7B), which were separate from vacuoles containing aleurain (Figure 7B).

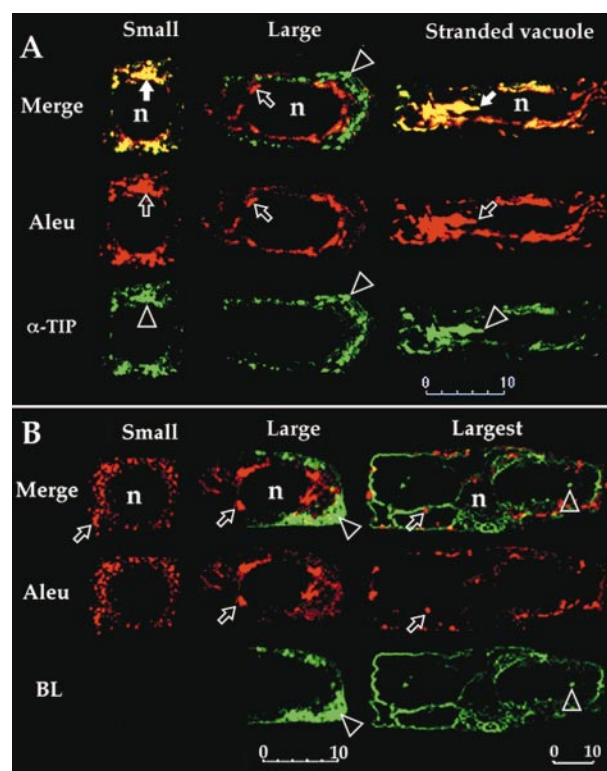


Figure 7. Comparison of Labeling for Aleurain with Labeling for Either Barley Lectin or α -TIP in Isolated Barley Root Tip Cells.

(A) Double labeling for aleurain (red) and α -TIP (green). Aleurain-containing vacuoles are indicated with open arrows, α -TIP-labeled vacuoles are indicated with open triangles, and colocalization of the two antibodies in merged images (yellow color) is indicated by solid arrows. See text for distinctions between smaller and larger cells and definitions of cells with stranded vacuoles.

(B) Double labeling for aleurain (red) and barley lectin (green). In 30 cells identified by positive staining for aleurain, aleurain-containing vacuoles (open arrows) and barley lectin-containing vacuoles (open triangles) were separate organelles. Presented are examples of labeling patterns obtained for three different cells distinguished by their sizes. Aleu, aleurain; BL, barley lectin; n, nucleus. Bars = $10 \mu\text{m}$.

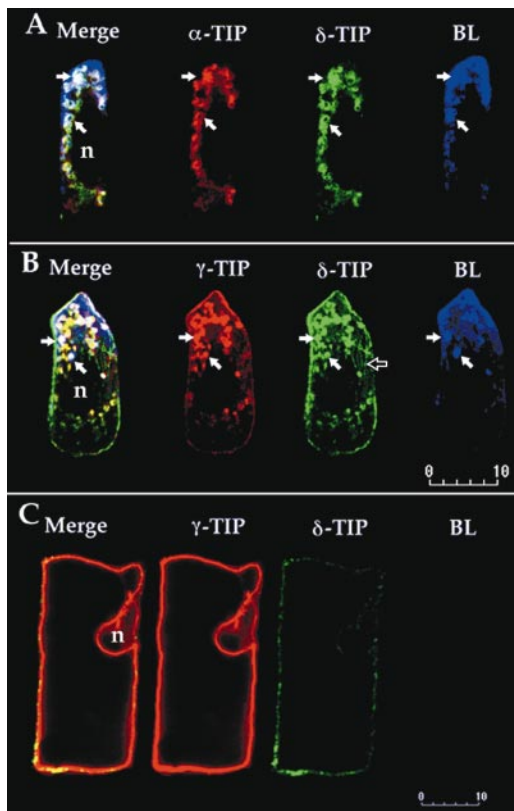


Figure 8. Immunofluorescent Triple Labeling for Barley Lectin and Different TIPs in Isolated Barley Root Tip Cells.

(A) Labeling for barley lectin (blue), α -TIP (red), and δ -TIP (green). Solid arrows indicate vacuoles labeled with the three antibodies; colocalization on the same organelles is indicated by white in the merged image.

(B) Labeling for barley lectin (blue), γ -TIP (red), and δ -TIP (green). Solid arrows indicate vacuoles labeled by the three antibodies, with colocalization indicated by white in the merged image. The open arrow indicates cortical microtubules that also were stained by the anti- δ -TIP peptide antibodies in barley cells.

(C) Example of an elongated cell with a large central vacuole: the central vacuole tonoplast stains strongly for γ -TIP, with only faint staining for δ -TIP and no staining for barley lectin.

BL, barley lectin; n, nucleus. Bars in **(B)** and **(C)** = 10 μ m.

Colocalization of α -TIP and δ -TIP on the PSV Tonoplast in Pea Seeds

The finding that barley lectin was present predominantly in vacuoles labeled with α -, δ - and γ -TIPs was unanticipated and led us to examine the association of different TIPs with PSVs in seeds. In pea cotyledons, the tonoplast of PSVs containing storage proteins was labeled with anti- α -TIP protein antiserum (Hoh et al., 1995). Using protein gel blot analysis with the anti-TIP peptide antibodies, extracts of mature pea seeds contained essentially only α - and δ -TIP (Figure

1B). When sections from seeds fixed and embedded in paraffin (Jauh et al., 1998) were studied using immunofluorescence labeling with the different anti-TIP peptide antibodies, cotyledon cells were filled with vacuoles that labeled strongly with both anti- α - and anti- δ -TIP antibodies (Figure 9). Presented are images from epifluorescence microscopy (Figure 9A), in which cell morphology from a differential interference contrast image can be used for reference to 4,6 diamidino-2-phenylindole staining for nuclear localization (upper right) and to PSV staining by anti- α -TIP (lower left) and anti- δ -TIP (lower right) antibodies. A confocal immunofluorescence image in which the optical section is out of the plane of the nucleus is shown in Figure 9B. A large PSV was stained by both antibodies, but the cytoplasm was filled with PSVs of various sizes that all were labeled with the two antibodies. Using our anti- δ -TIP peptide antibodies and the anti- α -TIP protein antibodies in immunoelectron microscopy studies of pea cotyledons, the Robinson group has demonstrated the presence of δ -TIP and α -TIP together on PSV tonoplast and on multivesicular body (thought to represent the PSV prevacuolar compartment) membranes (G. Hinz, S. Hillmer, and D.G. Robinson, personal communication). Therefore, although PSVs in pea cotyledons are invariably marked by the presence of α -TIP in their tonoplast, the presence of δ -TIP also appears to be necessary for their function (see Discussion).

Association of δ -TIP with a Specialized Vacuole Function

We hypothesize that δ -TIP plays an important role in establishing a storage function for vacuoles (see Discussion). In fully differentiated cells, δ -TIP alone is associated with specialized storage functions (Jauh et al., 1998). In root tip cells, however, δ -TIP is rarely found alone in the tonoplast (Table 1), and its association with other TIPs in vacuoles makes an evaluation of its role more complex. However, one functional property of vacuoles in root tip cells appears closely associated with the presence of δ -TIP.

The presence of arabinogalactan proteins in vacuoles has been suggested to result from endocytosis of the proteins from the cell surface (Herman and Lamb, 1992). We used monoclonal antibody CCRC-M7 (Steffan et al., 1995) in double-labeling experiments with the anti-TIP antibodies to determine if arabinogalactan was associated with any specific vacuole type. As shown in Figure 10, CCRC-M7 strongly stained cell walls as well as small punctate and spherical objects within some cells. Although these spherical structures were sometimes associated with α -TIP and γ -TIP (Figures 10A and 10B), in most instances they were separated from these vacuoles. However, these structures carrying the CCRC-M7 epitope were always within vacuoles carrying δ -TIP (Figure 10C). Thus, processes that result in accumulation of arabinogalactan within vacuoles appear to correspond closely with the presence of δ -TIP in the vacuole tonoplast.

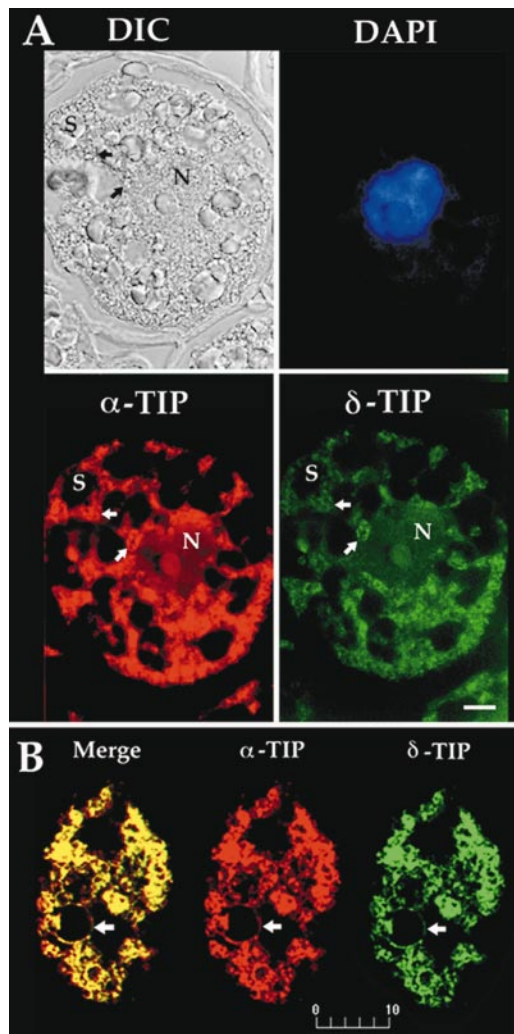


Figure 9. Colocalization of α - and δ -TIPs in the Tonoplast of PSVs in Pea Cotyledon Cells.

Mature, dry pea seeds were fixed, embedded in paraffin, and sectioned (Jauh et al., 1998). Images were recorded under differential interference contrast (DIC; [A]) or collected from sections stained with 4,6 diamidino-2-phenylindole (DAPI) (blue; [A]) anti- α - (red; [A] and [B]), and anti- δ -TIP (green; [A] and [B]) using lissamine rhodamine- and FITC-conjugated secondary antibodies, as described elsewhere (Jauh et al., 1998).

(A) Epifluorescence microscopy with a filter allowing simultaneous detection of red, green, and blue fluorescence was used (Jauh et al., 1998).

(B) The image was collected using laser scanning confocal microscopy.

Arrows show vacuoles with tonoplasts labeled by both antibodies, as demonstrated by the yellow color in the merged image. N, nucleus; S, starch grain. Bars in (A) and (B) = 10 μ m.

DISCUSSION

Interpretations of results from previous studies of TIP isoform expression patterns have been limited by the specificity of anti-TIP antisera used in those studies. As noted above (see Introduction), TIP-Ma27 antiserum (Marty-Mazars et al., 1995) identifies multiple proteins associated with vacuoles, and our results showed labeling with these antibodies in cell wall as well as in the lumen of vacuoles (Figure 4). In addition, antibodies raised against the purified α -TIP protein (Johnson et al., 1989) might well cross-react with " β -TIP" (Höfte et al., 1992). Recently, it was suggested that the anti- α -TIP antibodies could also cross-react with γ -TIP (Chaumont et al., 1998), although only conditions using large amounts of recombinant γ -TIP were tested.

Use of Anti-TIP Peptide Antibodies to Characterize Vacuoles

To identify protein gene products from individual members of the TIP gene family, we prepared antibodies to synthetic peptides representing the C-terminal cytoplasmic tails of α -, γ -, and δ -TIP. The antibody reagents would be specific for well-defined sequence epitopes and thus minimize uncertainties about the identities of proteins recognized. Their limitation would be the possibility that different members of a particular TIP isoform family might have amino acid changes within the epitope and therefore would not be recognized by an antibody. As demonstrated by protein gel blot and confocal immunofluorescence studies presented here and elsewhere (Jauh et al., 1998), each affinity-purified antipeptide antibody is highly specific for its particular TIP isoform, with no cross-reactivity toward other TIPs demonstrated under the conditions used. In addition, the antibodies appear to be specific for only TIP proteins, with only one exception identified: in root tip cells of barley, but not pea, the anti- δ -TIP antibodies appear to cross-react with cortical microtubules (e.g., Figure 8B).

The organelles labeled by the anti- α -, γ - and δ -TIP peptide antibodies ranged in size from $\sim 0.2 \mu$ m up to $>10 \mu$ m in diameter. All, however, were also labeled with anti-V-PPase antibodies, and the smallest organelles (e.g., Figure 2F) were labeled as strongly as the largest. It is well established that vacuole morphology and size can vary greatly, depending on the functional state (Campbell and Garber, 1980) or state of differentiation of a cell (Palevitz et al., 1981). Herman et al. (1994) identified 0.1- to 0.3- μ m "provacuoles" in root tip cells that carried vacuolar ATPase in their membranes; the small organelles labeled with the anti-TIP peptide and V-PPase antibodies in our studies may correspond to those structures.

Our results can be summarized as follows. In fully differentiated cell types, PSVs are marked by α -TIP plus δ -TIP, and lytic vacuoles are marked by γ -TIP. In contrast, in root

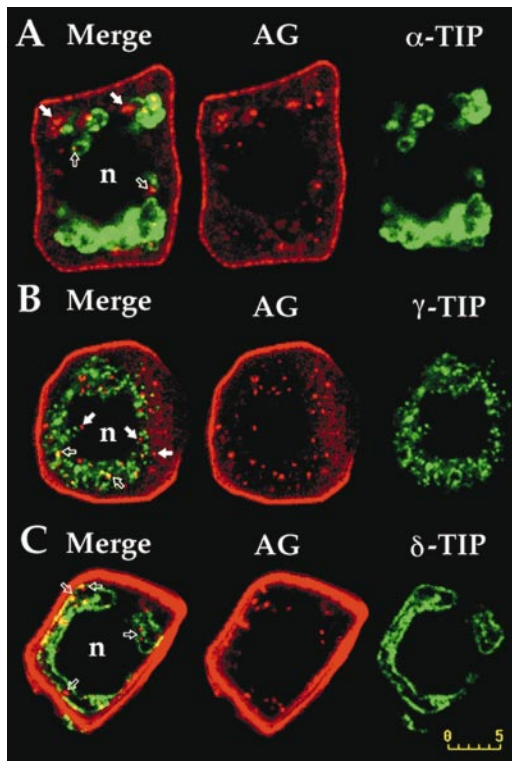


Figure 10. Double-Label Immunofluorescence with Anti-TIP Peptide and Arabinogalactan Antibodies in Isolated Pea Root Tip Cells.

(A) Hybridoma culture medium containing anti-arabinogalactan CCRC-M7 monoclonal antibody (1:10 dilution, red color) and anti- α -TIP antibody (1 μ g/mL, green color).

(B) Anti-arabinogalactan and anti- γ -TIP antibodies (1 μ g/mL, green color).

(C) Anti-arabinogalactan and anti- δ -TIP antibodies (2 μ g/mL, green color).

Open arrows indicate the presence of arabinogalactan within or closely associated with vacuoles labeled by anti-TIP antibodies, and solid arrows indicate arabinogalactan that is not associated with structures labeled by the designated anti-TIP antibody. AG, arabinogalactan; n, nucleus. Bar in (C) = 10 μ m for (A) to (C).

tips in which cells representing a wide range of different developmental stages are present, only infrequently were cells containing vacuoles marked by a single TIP identified. Consistent with previous results (Paris et al., 1996), barley lectin was almost exclusively found in vacuoles carrying α -TIP but in combination with δ -TIP or δ -TIP and γ -TIP. The association of aleurain with different TIP isoforms appeared to be highly dependent on the developmental stage and pathway followed by a given cell. For large, elongated cells, the large central vacuole contained aleurain and was marked by γ -TIP alone. For small cells with numerous small vacuoles, aleurain-containing vacuoles were marked by δ -TIP or δ -TIP and γ -TIP in most instances. Two specific exceptions to this rule

were observed, however. For small square cells that were most likely derived from the meristem, aleurain was present in vacuoles that did not contain barley lectin but were marked by α - plus δ -TIP. A second, infrequently observed cell type was elongated with vacuoles forming a network of strands that extended the full length of the cell; these vacuoles contained aleurain and were also marked by α -TIP. We hypothesize that in cells with a single vacuole type, that is, cells early in a pathway of differentiation, the single vacuole is marked by α - plus δ -TIP (Figure 11A). As vacuoles with different functions develop, however, the pattern changes. The results in aggregate are consistent with a model (Figure 11B) in which α -TIP in the presence of δ -TIP or δ -TIP and γ -TIP defines PSVs, which represent the terminus of the smooth dense vesicle pathway (Neuhaus and Rogers, 1998). The second vesicle pathway, that for clathrin-coated vesicles (Jauh et al., 1998; Neuhaus and Rogers, 1998), leads to vacuoles marked by δ -TIP, γ -TIP, or δ -TIP and γ -TIP. The presence of δ -TIP indicates a storage function, whereas γ -TIP alone defines the lytic vacuole on this pathway.

Thus, δ -TIP is associated with storage functions in both the PSV and lytic vacuole branches of the vacuolar system, and we hypothesize that in the absence of δ -TIP, either type of vacuole may have a lytic function. Swanson et al. (1998) have previously proposed an association of α -TIP and autophagic vacuoles in barley aleurone protoplasts. Aleurain may be present in vacuoles with any of the three TIP combinations, δ -TIP, γ -TIP, or δ -TIP and γ -TIP, but we hypothesize that only the γ -TIP vacuole is a lytic vacuole (Figure 11B). This hypothesis is based on (1) the observation that delta vacuoles, with δ -TIP alone in their tonoplast, store vegetative storage proteins and pigments, whereas γ -TIP vacuoles apparently acquire the capacity to store vegetative storage proteins coincident with the appearance of δ -TIP in their tonoplast (Jauh et al., 1998); and (2) the fact that, in fully elongated root tip cells, the large central vacuole containing aleurain is labeled predominantly or exclusively by anti- γ -TIP.

A Model for Biogenesis of Different Types of Vacuoles

We tried to identify meristematic cells in our root tip cell preparations so that we would know what vacuole types were present at the earliest stages of cell differentiation. Cells that would most likely qualify as meristematic are those we identified as small (e.g., Figures 3B, 3E, 7A, and 7B, left), which we specifically identified within populations expressing aleurain. The patterns of TIP expression within the small cells can be derived from those results (Figure 6). Approximately one-third of the cells in which aleurain was found in δ -TIP vacuoles were of this small class (Figure 6A). The results presented in Figure 7B show that where aleurain was associated with α -TIP, the aleurain-containing α -TIP vacuoles were also predominantly in the small cells. We could not perform triple labeling to prove that the aleurain-containing vacuoles in small cells were marked by both

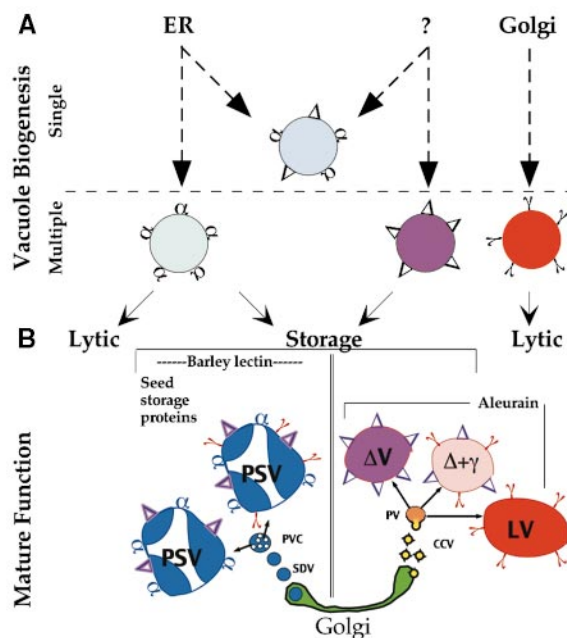


Figure 11. TIP Isoforms as Markers for Vacuolar Functions.

(A) Vacuole biogenesis. Greek letters surrounding each vacuole type indicate which TIPs are present in the tonoplast. “Single” indicates the proposed vacuole type in meristematic cells with only a single vacuole, and “multiple” indicates relatively undifferentiated cells with more than one vacuole type. Arrows with dashed lines indicate proposed flow of membrane to organize different vacuoles. Because small, putatively meristematic cells appear to have only one type of vacuole that is marked by $\alpha + \delta$ -TIP, it is possible that this represents the single vacuole present at the earliest stage of differentiation and is thus positioned highest in the sequence. Data presented in the text, however, argue that three separate vacuole types are formed subsequently, indicated by α , Δ , and γ .

(B) Mature functions. This diagram divides the vacuolar system into two parts: vacuoles marked by α -TIP in their tonoplast are to the left of the double line, and vacuoles lacking α -TIP are to the right. Bold-face letters identify functionally distinct vacuoles: PSV, protein storage vacuole; ΔV and $\Delta + \gamma$, delta vacuoles (Jauh et al., 1998); LV, lytic vacuole. Above the diagram are indicated the likely general functions for the vacuoles, where LV is a lytic compartment and the central four have storage functions. Below the bar indicating storage vacuoles are noted molecules that have been identified in each type of storage vacuole. To the right, lines bracketing “Aleurain” indicate that this protease may be localized within each of the three vacuoles. Below the vacuoles are depicted two separate vesicular pathways that originate from the Golgi: (1) Using a receptor-mediated process, clathrin-coated vesicles (CCV) carry soluble proteins to prevacuoles (PV; Paris et al., 1997) that are proposed then to fuse with any of the three types of non- α -TIP-containing vacuoles. (2) Smooth dense vesicles (SDV) deliver seed-type storage proteins to PSVs via a prevacuolar compartment (PVC) that may be comprised of multivesicular bodies (Robinson et al., 1998; Hinz et al., 1999).

α - and δ -TIP because the antialeurain, anti- δ -TIP, and anti- α -TIP antibodies were all polyclonal rabbit antibodies. This, however, is the simplest explanation for the results presented in Figure 6 and fits well with results from small pea root tip cells studied with anti-TIP antibody triple labeling (Figures 3B and 3E).

From this limited amount of information, we hypothesize that cells in the root meristem begin with one vacuole type that contains aleurain and whose tonoplast is marked by α - and δ -TIP; this is indicated in Figure 11A with the single vacuole above the dashed line. As cell differentiation occurs, presumably more complex cellular processes would require specialized vacuole functions, and expression of γ -TIP would be associated with the development of individual organelles in which α -, δ -, and γ -TIP could be localized separately (Figure 11A). The cell shown in Figure 3E exhibits some features that would fit with this model. α -TIP labels the tonoplast on relatively large vacuoles, and smaller spherical structures labeled with δ -TIP are predominantly colocalized to α -TIP-labeled tonoplast (as indicated by yellow color), although clear examples of δ -TIP vacuoles separate from α -TIP are indicated. In contrast, small vacuoles marked by γ -TIP are predominantly separate from α - and δ -TIP, although the three TIPs are occasionally localized together on the same structure (as indicated by the white color). According to our model, this cell would represent a transient stage occurring immediately after the earliest stage with a single α - plus δ -TIP vacuole; γ -TIP has been expressed, and functionally distinct vacuoles marked by individual TIP isoforms are being organized. Importantly, however, the presence in this cell of vacuoles marked by a single one of each of the three TIPs indicates that a cell can form and maintain three separate vacuole types. Thus, it appears that the cell does not simply make a vacuole in which δ - and/or γ -TIP is sent by default and then modify it in some cases by the further addition of α -TIP.

How cells change vacuoles marked by individual TIPs (Figure 11A) to make functionally mature vacuoles marked by combinations of TIPs (Figure 11B) is unknown, but this process would probably involve some type of fusion of the different precursors. This question is closely related mechanistically to the question of how a cell forms and maintains individual organelles. The fact that two separate vesicular pathways traffic to the two sides of the vacuolar system (Figure 11B) clearly indicates that the cell regards α -TIP-containing and non- α -TIP-containing vacuoles as separate organelles, but it is likely the TIP isoforms themselves do not define organelle identity.

Organelle identity has been hypothesized to be dependent on the presence of an organelle-specific syntaxin. Thus, the *Saccharomyces cerevisiae* genome contains genes for eight syntaxin homologs, with one specific for endoplasmic reticulum, one specific for traffic to *cis*-Golgi, two that correspond to yeast equivalents of the *trans*-Golgi network and early endosome, respectively, one for the prevacuolar compartment, one for the vacuole, and two on the

plasma membrane (Holthuis et al., 1998; Nichols and Pelham, 1998). Under this model, the plant vacuolar system as depicted in Figure 11B would then require a minimum of four syntaxins to define the proper organelles: one each for the lytic prevacuole, the delta vacuole/lytic vacuole, the PSV prevacuolar compartment, and the PSV. To fit our observations within this model, we postulate that an organelle-specific syntaxin for each functionally distinct vacuole would allow delivery of the proper TIP isoform to the organelle but that the TIP isoform would be responsible for defining the function of the vacuole by somehow modifying the environment within the vacuole lumen.

Much more information about vacuole biogenesis and how developmental pathways affect that process is needed. As more antibody markers for specialized vacuole functions, such as transporters for metabolic products or conjugated compounds, become available and as more biochemical details about the composition of different vacuole tonoplasts are accumulated, it should be possible to refine this model and provide a better explanation for the biogenesis and maintenance of these unique organelles.

METHODS

Primary and Secondary Antibodies

Rabbit antisera raised against pea tonoplast intrinsic protein α -TIP (Johnson et al., 1989) and against radish γ -TIP protein (Maeshima, 1992) were provided by K. Johnson (San Diego State University, CA) and M. Maeshima (Nagoya University, Nagoya, Japan), respectively. M. Maeshima also provided antiserum to purified mung bean V-PPase (Maeshima and Yoshida, 1989). Antiserum 324 raised against a synthetic peptide representing putative hydrophilic loop IV (Sarafian et al., 1992) was provided by P. Rea (University of Pennsylvania, Philadelphia). Monoclonal antibody CCRC-M7 raised against arabinogalactan was generously provided by M. Hahn (University of Georgia, Athens) (Steffan et al., 1995). The production of synthetic peptides representing the C-terminal sequences of α -, γ -, and δ -TIP and dark-induced protein (DIP; Culiarez-Macia and Martin, 1993) and their related affinity-purified antipeptide polyclonal antibodies were described previously (Jauh et al., 1998). The monoclonal antibody raised against the γ -TIP C-terminal sequences was produced by the Washington State University (Pullman, WA) monoclonal antibody facility using methodologies similar to those described by Rogers et al. (1997).

Affinity-purified goat anti-wheat germ agglutinin (WGA) antibodies were purchased from Vector Laboratories (Burlingame, CA) and used at a concentration of 10 μ g/mL. The amino acid sequences of WGA and barley lectin are highly conserved, and these two proteins are immunologically indistinguishable (Lerner and Raikhel, 1989). The anti-WGA antibody was therefore used to detect barley lectin in barley root tip cells (Paris et al., 1996). Affinity-purified rabbit antialeurain antibodies were prepared as previously described (Holwerda et al., 1990) and used at a concentration of 10 μ g/mL. All fluorochrome-tagged secondary antibodies, such as lissamine rhodamine, Cy-5, and fluorescein isothiocyanate (FITC), were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) and were used at a 1:200 dilution, except the anti-rabbit F(ab')₂ fragment conjugated

with lissamine rhodamine, which was used at a 1:20 dilution in double- or triple-labeling experiments.

Immunofluorescence Staining

Pea and barley root tip cells were isolated as described previously (Paris et al., 1996). Briefly, root tips from 3-day-old germinating pea seedlings and 2-day-old germinating barley seedlings were cut and fixed for at least 24 hr in 3.7% paraformaldehyde in 50 mM sodium phosphate buffer, pH 7.0, and 5 mM EGTA. To disperse single cells, we treated the fixed root tips with 3% Cellulysin (Calbiochem, La Jolla, CA) for 20 min to partially digest cell walls. Single cells were released by gently squeezing the digested root tips between two microcentrifuge tubes. The plasma membranes of isolated cells were permeabilized with 0.5% Triton X-100 in 50 mM sodium phosphate buffer, pH 7.0, and 5 mM EGTA for 5 min. The cells were then incubated with blocking buffer (PBS containing 0.25% BSA, 0.25% gelatin, 0.05% Nonidet P-40, and 0.02% sodium azide) at room temperature for 30 min.

The methods for immunofluorescent labeling with two different rabbit polyclonal antibodies have been described previously (Paris et al., 1996). After treatment with blocking buffer, the root tip cells were incubated at room temperature for 1 hr with the first primary antibodies, either one polyclonal antibody preparation alone for double labeling or one polyclonal plus one monoclonal antibody preparation for triple labeling. After washing with blocking buffer for 30 min, the cells were incubated with secondary antibodies at room temperature for 1 hr: an excess amount (1:20 dilution) of goat anti-rabbit F(ab')₂ fragments conjugated with lissamine rhodamine plus a 1:200 dilution of anti-mouse IgG coupled to Cy-5 for triple labeling. Cells were incubated with blocking buffer for 30 min to wash out unbound secondary antibodies. Cells were then postfixed with 3.7% paraformaldehyde (in 50 mM phosphate buffer, pH 7.0, and 5 mM EGTA) at room temperature for 1 hr and then incubated in blocking buffer overnight at 4°C. The postfixed cells were treated with blocking buffer at room temperature for 1 hr and then incubated with the appropriate second rabbit polyclonal antibody preparation at room temperature for 2 hr. Control cells were incubated with blocking buffer only. After washing with blocking buffer at room temperature for 30 min, the cells were incubated at room temperature for 1 hr with a 1:200 dilution of the secondary anti-rabbit antibodies conjugated with FITC. After washing, cells were mounted in Mowiol (Calbiochem).

Confocal Laser Scanning Microscopy and Data Processing

Labeled cells were examined by laser scanning confocal microscopy. Images were recorded using a Bio-Rad MRC-1024 2PMT system with an inverted compound microscope (model TE300; Nikon Inc., Melville, NY) using a Planado \times 60 (numerical aperture = 1.4) oil immersion objective (Nikon). Fluorochrome-labeled samples were excited with a neutral density filter that used either 3 or 1% of the laser power to ensure that the signal was within the linear range of detection. The images were collected sequentially from the same optical section with either Cy-5/rhodamine or Cy-5/rhodamine/FITC programmed software for double and triple labeling, respectively. The recorded images were processed by Adobe Photoshop 4.0 software, as previously described (Paris et al., 1996). For double labeling, the Cy-5 images were pseudocolored in green, the rhodamine

images in red, and the merged images in yellow. For triple labeling, the Cy-5 images were pseudocolored in blue, the rhodamine images in red, and the FITC images in green.

Protein Extraction and Immunoblot Analysis

For dot immunoblots, defined amounts of synthetic peptides conjugated to BSA were applied to a nitrocellulose membrane and blocked with PBS containing 5% nonfat milk (w/v), 0.02% Tween 20, and 0.02% sodium azide (PBST) at room temperature for 30 min. Blots were then incubated with primary antibodies (in PBS) at room temperature for 2 hr. After washing for an hour with six changes of PBST, the blots were incubated with secondary antibodies, 1:7500 dilution of goat anti-rabbit (Sigma) or anti-mouse (Jackson ImmunoResearch Laboratories) IgG-alkaline phosphatase conjugates. Detection of antibody conjugates was as described previously (Rogers et al., 1997). For protein gel blots, total proteins were extracted from pea root tips, pea seeds, petunia petals, radish roots, and soybean leaves by homogenization at 25°C in 0.0625 M Tris-HCl, pH 6.0, and 2% SDS with a polytron-type homogenizer (model PRO250; PRO Scientific, Monroe, CT). After homogenization, the extracts were allowed to incubate for 1 hr at 25°C. The supernatants were then obtained by centrifugation at 12,000g at 25°C for 20 min. The tissue extracts were separated on 12.5% SDS-polyacrylamide gels and transferred to nitrocellulose, as previously described (Jauh et al., 1998). Incubation with antibodies and color development were as described above.

Electron Microscopy

Lateral roots of 6-day-old plants were immersed in 20% dextran (molecular mass of ~39 kD) and frozen under high pressure in a Balzers HPM 010 freezing apparatus (generously performed by L. Andrew Staehelin and Tom Giddings, University of Colorado at Boulder).

After shipment to the University of Missouri in liquid nitrogen, the frozen samples were transferred to 2% osmium tetroxide in anhydrous acetone at -78°C for 5 days. The samples were then slowly warmed to -20°C over several hours and finally thawed to 4°C by using a freeze-substitution and infiltration protocol similar to that of Zhang and Staehelin (1992). After 2 hr at 4°C, the samples were rinsed once in acetone at 4°C and then for 4 hr in 100% methanol at 4°C to remove some of the phospholipids and improve resin infiltration (Zhang and Staehelin, 1992). The samples were then returned to ice-cold acetone and allowed to warm slowly to room temperature. A slow infiltration in London Resin White embedding medium (Electron Microscopy Sciences, Fort Washington, PA) was accomplished by gently rotating the samples in a mixture of 10% resin in acetone for 24 hr. On each subsequent day, the resin concentration was increased by 10% until the samples were in 100% resin. After four 24-hr rinses in fresh 100% resin, the samples were popped off the brass freezing stages, placed in fresh resin in BEEM capsules (Electron Microscopy Sciences), and polymerized at 52°C for 24 hr.

Silver sections were picked up on 600-mesh nickel grids and incubated for 10 min in blocking buffer (0.25% BSA, 0.25% gelatin, 0.25% normal goat serum plus 0.08% Micr-O-protect [Roche Molecular Biochemicals, Indianapolis, IN]) in Hepes wash buffer (70 mM NaCl, 30 mM Hepes, and 2 mM CaCl₂, pH 7.4) and then for 1 to 2 hr at room temperature on a 40-μL drop of a 1:100 dilution of the pri-

mary antibody preparation. After extensive rinsing, the grids were placed on a 40-μL drop of a 1:25 dilution of affinity-purified goat anti-rabbit IgG conjugated to 12-nm colloidal gold (Jackson ImmunoResearch Laboratories) for 1 to 2 hr at room temperature. After rinses in Hepes wash buffer, sections were counterstained by floating them sequentially on droplets of 2% osmium tetroxide for 20 min, 8% uranyl acetate for 20 min, and Reynold's lead citrate (Electron Microscopy Sciences) for 3 min with rinsing in distilled water after each staining step.

Control experiments included staining sections in an identical fashion with either a nonimmune rabbit serum or an affinity-purified antiserum against a *Vibrio cholerae* capsular antigen for 2 hr followed by a 2-hr incubation with a 1:25 dilution of the gold-conjugated secondary antisera. With the exception of a rare isolated gold particle, no staining was observed in the absence of the anti-TIP antibodies.

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