Newly Formed Vacuoles in Root Meristems of Barley and Pea Seedlings Have Characteristics of both Protein Storage and Lytic Vacuoles

Andrea Olbrich, Stefan Hillmer, Giselbert Hinz, Peter Oliviusson, and David G. Robinson*

Dept. Cell Biology, Heidelberg Institute for Plant Sciences, University of Heidelberg, 69120 Heidelberg, Germany

* Corresponding author; e-mail david.robinson@urz.uni-heidelberg.de; Fax: +49-6221-546404

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is David G. Robinson (david.robinson@urz.uni-heidelberg.de)

Running Title: Root tip vacuoles
Abstract

Plant cells are considered to possess functionally different types of vacuole in the same cell. One of the papers cited in support of this concept is that of Paris et al. (2001 Cell 85: 563-572) who reported that protein storage and lytic vacuoles in root tips of barley and pea seedlings were initially separate compartments which later fused to form a central vacuole during cell elongation. We have reinvestigated the situation in these two roots using immunogold electron microscopy as well as immunofluorescence microscopy of histological sections. Using antisera generated against the whole protein of α−TIP as well as specific C-terminal TIP peptide antisera against α−, γ−, and δ-TIP, together with antisera against the storage proteins barley lectin, and pea legumin and vicilin, we were unable to obtain evidence for separate vacuole populations. Instead, our observations point to the formation of a single type of vacuole in cells differentiating both proximally and distally from the root meristem. This is a hybrid type vacuole containing storage proteins and having both α− and γ− TIPs, but not δ-TIP, in its tonoplast. As cells differentiate towards the zone of elongation their vacuoles are characterized by increasing amounts of γ−TIP, and decreasing amounts of α−TIP.

INTRODUCTION

Most differentiated plant cells are dominated by the vacuole, an organelle that by storing sugars, inorganic ions, organic acids and secondary metabolites serves to generate and regulate cell turgor (De, 2000). The various functions of the vacuole are highly tissue-specific (Marty, 1999; Tomos et al., 2000), whereby a distinction is generally made between lytic vacuoles (LV) which harbor hydrolytic enzymes, and protein storage vacuoles (PSV) in which non-enzymic proteins accumulate. PSV are, however, compound organelles since they contain specific hydrolytic enzymes required for storage protein processing (Müntz and Shutov, 2002), as well as crystalline inclusions (Jiang
et al., 2000). LV also have vacuolar processing enzymes (VPE), but these belong to a genetically different class of proteins, than those present in the PSV (Hara-Nishimura and Maeshima, 2000). As might be expected, PSV are usually found in organs where proteins are stored in large amounts e.g. seeds, especially of legumes (Pernollet, 1978; Bewley and Black, 1994). However, in response to wounding (depodding), storage proteins may also accumulate in vacuoles in vegetative tissues (Franceschi et al., 1983; Staswick, 1994). More recently, PSV-type vacuoles have also been detected in mesophyll cells (Di Sansebastiano et al., 1998; Park et al., 2004).

In addition to lumenal contents, vacuoles may also be distinguished from one another on the basis of specific isoforms of tonoplastic intrinsic proteins (TIPs, Johnson et al. 1989; Höfte et al. 1992; Chrispeels et al., 1997), which belong to the superfamily of aquaporins (Preston et al., 1992; Chrispeels and Maurel, 1994). Thus, α-TIP is present in the tonoplast of the PSV in bean seeds (Johnson et al., 1989), while γ-TIP is typical of LV and is highly expressed in elongating Arabidopsis root cells (Ludevid et al., 1992). Polyclonal antisera generated against the complete TIP molecules have been successfully used on numerous occasions to identify LV and PSV membranes in subcellular fractions by Western blotting (Hoh et al., 1995; Barrieu et al., 1998), and in situ by immunogold electron microscopy (Hoh et al. 1995; Barrieu et al., 1998; Serraj et al., 1998; Hinz et al., 1999; Fleurat-Lessard et al., 1997; 2005). More recently antibodies raised against specific peptides at the non-conserved C-tail of the TIP molecule (Jauh et al., 1999; Jiang et al., 2001), have also proved to be of diagnostic value.

A number of studies have shown that different types of vacuole can co-exist in the same cell. Some of these rely on obvious visible differences in the lumen of the vacuoles in the electron microscope (e.g. Fleurat-Lessard, 1988; Hoh et al., 1995). In other investigations, the variable accumulation of expressed GFP-tagged soluble vacuolar markers (aleurain-GFP for LV-type vacuoles; GFP-chitinase for PSV type vacuoles) has been used as an indicator for different populations of vacuoles (Di Sansebastiano et al., 1998; 2001). Interestingly, however, the targeting of a particular soluble vacuolar marker to the large central vacuole, or to smaller vacuoles in the cell cortex,
seems to vary from cell type to cell type, especially in leaves (Flückiger et al., 2003). These differences may well relate to vacuolar pH with some vacuoles being neutral and others acidic (Swanson et al., 1998; Diwu et al., 1999; Epimasko et al., 2004).

The most frequently cited paper in support of what is sometimes termed the “two-vacuole hypothesis” is a paper from Paris et al. (1996), which deals with the identification of different vacuole populations in root tips. Since roots have meristems whose cells have no or only small vacuoles, followed distally by a zone of elongation which is functionally correlated with vacuolar expansion, this is a classical organ for studies on vacuole biogenesis and vacuole development (Marty, 1999; De 2000). Until publication of the paper of Paris et al. (1996), vacuoles in the primary root - being vegetative tissue - were generally considered to be of the lytic (LV) type. Using \( \alpha \) and \( \gamma \) antisera as membrane probes, and antibodies against barley lectin and aleurain as content markers, Paris et al. (1996) demonstrated the presence of PSV in addition to LV in the roots of barley and pea. According to these authors, the two vacuole types were separate compartments in the immediate post-meristematic cells and later fused with one another to form the large central vacuole as the cells differentiate upwards in the root.

We have reinvestigated the distribution of TIPs and content markers in the roots of barley and pea using immunofluorescently labeled sections from Steadman wax embedded samples, and immunogold electron microscopy. In contrast to Paris et al. (1996), whose observations were made on squash preparations where cell position in the root cannot be accurately determined, our results do not support the existence of two initially separate vacuole populations. Instead, post-meristematic cells both in the calyptra and upwards in the root form only one type of vacuole: a PSV. As differentiation proceeds, \( \alpha \)TIP is gradually replaced by \( \gamma \)TIP in the membrane of the enlarging vacuole. The production of a PSV rather than a LV as the primary vacuole type in post-meristematic cells is most likely related to seed-specific signals still being dominant in the meristem of the primary root.
RESULTS

Detection of Storage Proteins and TIPs in Barley and Pea Roots

We isolated total membrane fractions from 1 mm segments sequentially excised from barley and pea root tips, separated the proteins by SDS-PAGE and carried out Western blotting with TIP antiserum and antibodies against barley lectin (for barley roots) and vicilin and legumin (for pea roots). In barley roots, both \( \alpha \)- and \( \gamma \)-TIPs were found to be present in all root tip segments (Fig. 1 A, B). For the detection of \( \alpha \)-TIP, polyclonal antibodies directed against the whole protein (Johnson et al., 1989) as well as the C-terminal peptide (Jauh et al., 1998, 1999) were used. Similarly for \( \gamma \)-TIP a peptide antibody (Fig. 1 B; Jauh et al., 1998), and a whole protein antibody (VM 23, Maeshima, 1992; data not shown) were used, and give identical results. However, \( \delta \)-TIP which, together with \( \alpha \)-TIP, is typical of the boundary membrane of protein bodies from pea cotyledons (Fig. 1 E), was not detected by the \( \delta \)-TIP peptide antiserum of Jauh et al. (1998) in barley root tips. Barley lectin was also found to be present in all root tip segments (Fig. 1 C). \( \alpha \)- and \( \gamma \)-TIPs were detected in all segments of the pea root tip (Fig. 1 E), as were vicilin and legumin – the principal storage globulins of pea seeds. Interestingly, these globulins were not proteolytically processed into mature storage proteins, as they are in developing cotyledons. Thus, only prolegumin polypeptides with molecular mass around 60 kDa (Hinz et al., 1999) were detected in root tips of pea.

Cellular Distribution of Barley Lectin and TIPs in Barley Roots

Barley lectin is not uniformly expressed throughout the root tips of germinating barley, neither in 3 day nor 10 day old roots (Fig. 2 B-D; Suppl. Fig. 1 A). Whereas all cells of the calyptra stained positively for barley lectin (Fig. 2 B-D), its synthesis in the rest of the root tip is restricted to the rhizodermis and the outermost 2-3 cell layers of the cortex (Fig. 2 B, C; Suppl. Fig. 1 A). No lectin signal was visible in the cells of the central cylinder. Interestingly, whereas the immunostaining pattern for \( \alpha \)-TIP was similar to that for barley
lectin in the non-calytra portion of the root tip, only the cells surrounding the
columella of the calyptra stained positively for α-TIP (Fig. 2 E-H). In root tips
from older seedlings the α−TIP signal is particularly strong in the cells of the
meristem (Suppl. Fig.1 B). In addition, the cells of the central cylinder are also
labeled, but more weakly. The distribution of γ-TIP in 3 day old roots was
remarkably similar to that of barley lectin: all cells of the calyptra, and only the
rhizodermis and outermost cortex cells reacting positively (Fig. 3 A – C). In
comparison, in 10 day old roots, cells of the central cylinder gave a weak
signal for γ-TIP, but there was almost no signal in the rhizodermis (Suppl. Fig.
1 C).

**Cellular Distribution of Vicilin and TIPs in Pea Roots**

In root tips of 3 day old pea seedlings, labeling with vicilin antibodies was
seen in the meristem, cortex and extreme tip of the calyptra, but neither in the
central cylinder nor the rest of the calyptra (Fig. 4 B). In 10 day old root tips,
vicilin and α−TIP were no longer detected (Suppl. Fig. 1 D, E). In pea root
tips, γ−TIP labeling was observed in the outermost cells of the root cap, and
the innermost layers of the cortex above the meristem, while the meristem
itself, the majority of the calyptra and the rhizodermis were without signal (Fig.
5 A – C). In 10 day old root tips, the γ- TIP signal resembled that of 3 day old
root tips, with the strongest signal being in the innermost cells of the cortex
(Suppl. Fig. 1 F).

**Immunogold Electron Microscopy (IEM) of Barley and Pea Root Tips: No
Evidence for Separate Vacuole Populations**

We have performed IEM on barley root tips with antibodies generated against
the storage protein barley lectin as a content marker, and with antibodies
against α- and γ-TIP. We selected cells in those areas which stained
positively for barley lectin by immunofluorescence: cells immediately
bordering on the meristem, cells in the cortex on the flanks, and cells in the
calyptra. The vacuoles in all cells investigated had electron opaque deposits,
which stained positively with barley lectin antibodies (Fig. 6 A-D). In cells in the cortical flank (Fig. 6 C), or in the calyptra (Fig. 6 A) large lectin-positive deposits, sometimes in the middle of the vacuole lumen, were regularly observed. In cells immediately surrounding the quiescent center where vacuole formation begins we observed vacuoles with internal membranes in addition to lectin-positive protein aggregates (Fig. 6 B-D). Such structures are typical for vacuoles which arise from an initially cisternal-tubular system by a progressive, inwardly directed, dilation, as previously described for PSVs in developing pea cotyledons (Hoh et al., 1995). An early stage in this process where an almost complete tire-like provacuole with protein aggregates is seen surrounding what is sometimes termed a “zone of exclusion” (Amelunxen and Heinze, 1984; Robinson and Hinz, 1997) is presented in Fig. 6D.

Vacuoles with storage protein aggregates in barley roots stained positively with $\gamma$-TIP antibodies in single immunogold labeling preparations (Fig. 7 B). We also performed a variant of double immunogold labeling by which different sides of the section were exposed to a different antibody. In this way we were able to demonstrate the presence of both $\alpha$- and $\gamma$-TIPs in the membrane of PSVs present in cells from the cortical flank (Fig. 7 A, D) and the calyptra (Fig. 7 C, D).

Vacuoles from cells in the cortical flanks distal to the meristem of pea roots which reacted positively towards vicilin antibodies in immunofluorescence, also contained vicilin-positive storage protein aggregates (Fig. 8 A). These vacuoles also labeled positively with both $\alpha$- and $\gamma$-TIP antibodies (Fig. 8 B). In double-immunogold labeled sections we could not find vacuoles which were labeled exclusively by only one TIP antibody.

**DISCUSSION**

The classical notion of the vacuole as being a multifunctional single organelle in the plant cell (Wink, 1993), has gradually been superseded over the last decade by a multi-compartment concept with different vacuole types coexisting in the same cell (Robinson and Rogers, 2000). Not only has
convincing morphological data been presented for functionally distinct vacuoles (for literature see: Epimashko et al., 2004; Neuhaus and Paris, 2006), but indirect evidence for the operation of different transport routes for storage proteins and acid hydrolases also exists (Vitale and Hinz, 2005; Hinz et al., 2007; Sanmartin et al., 2007). Especially important for the development of the multivacuolar concept has been the discovery of vacuole-specific isoforms of tonoplast intrinsic proteins (TIPs, Johnson et al., 1989; Höfte et al., 1992; Ludevid et al., 1992). These allow lytic-type vacuoles to be distinguished from vacuoles in which proteins are stored, especially in seeds. Actually, the TIP isoforms are very similar and differ only in their C-terminal cytoplasmic tails, against which specific peptide antibodies have been generated (Jauh et al., 1998; 1999). In immunofluorescence studies, together with antibodies against content proteins e.g. the cysteine protease barley aleurain for LVs (Paris et al., 1996; Jauh et al., 1999), vegetative storage proteins and protease inhibitors for vegetative PSVs (Jauh et al., 1998), and barley lectin and chitinase for seed PSVs (Jiang et al., 2000), it has been possible to allocate the TIP isoforms to specific vacuole types. Thus, according to Jauh et al. (1998; 1999) PSVs in vegetative tissues are basically characterized by δ-TIP, whereby seed PSVs may have in addition α− or γ-TIP. In contrast LVs have only γ-TIP, and autolysosomes – induced by starvation or treatment with the cysteine protease inhibitor E-64 – have only α− TIP in their membranes (Moriyasu et al., 2003).

A key paper in support of the multivacuole concept is that of Paris et al. (1996), which described the existence of two separate populations of vacuoles in root tips of barley and pea seedlings. These two vacuoles: a PSV marked by the presence of barley lectin and α-TIP, and a LV with aleurain and γ-TIP were reported to be present in cells immediately derived from the meristem, but as cell growth continued they fused with one another to form a large central vacuole. Unfortunately, the data of Paris et al. (1996) was essentially obtained on individual cells released by enzymatic maceration, so that for all double labelings the original position of the cells in the root could not be ascertained. Paris et al. (1996) did present immunofluorescent data from histological sections of root tips, but only for single TIPs in different
organisms: α−TIP in pea and barley, γ−TIP in pea. In agreement with their results, we also found that both TIPs, in both root tips, are not equally expressed in all tissues, although this does appear to be the case with V-ATPase (Suppl. Fig. 2). In particular, cells in the central cylinder (the stele) showed weak or no signals for either α− or γ−TIP as well as the storage proteins barley lectin and vicilin. This result was confirmed in the electron microscope: the tonoplasts of cells in the central cylinder did not react positively to any of the antisera (data not shown). Exactly what kind of TIP is expressed in the vacuoles of the central cylinder remains a matter for speculation. We should, however, not fail to point out that our observations and those of Paris et al. (1996) stand in contradiction to those of Höfte et al. (1992), since the latter authors presented Western blots showing that α−TIP expression was restricted to the embryo and endosperm in Arabidopsis and did not occur in the roots. The reason for this difference is unclear, but it cannot be a question of the antisera (peptide antibodies versus antibodies raised against the total TIP protein) since the original α-TIP antiserum of Johnson et al. (1989) which was used by Höfte et al. (1992) also gave a positive signal in barley roots (see Fig. 1).

The detection of vicilin and legumin, albeit as unprocessed proforms, in the vacuoles of pea root tips is a significant observation. The presence of these seed tissue globulins in non-storage tissues i.e. roots, during seed germination seems at first glance surprising. The question is whether this reflects the continued expression of seed globulin genes during germination, or merely the presence of residual globulins synthesized during embryogenesis? It has been previously demonstrated that vicilin and legumin are not only expressed during embryogenesis, but also during microsporogenesis (Zakharov et al., 2004), and this has been interpreted as a response to the need for reserves of amino acids required during seed and pollen germination (Hall et al., 1999). However, it has also been shown that promoters for numerous storage globulins are precisely regulated, being highly active during embryogenesis, and then being switched off during all subsequent phases of vegetative development (Chandrasekharan et al., 2003). Therefore, it would seem that the presence of storage proteins in root
tip cells of 3 day old pea seedlings is not a result of new synthesis during germination, especially since they are no longer detectable after 10 days of germination. In this regard, the study of Tiedemann et al. (2000) is worthy of mention. They reported that during embryogenesis in vetch (*Vicia sativa*), storage globulins are expressed both in the cotyledons and the embryonic axis (which gives rise to the radicle and subsequently the root). Interestingly, the storage globulins in the embryonic axis are consumed during the first 2-3 days of germination, since the mobilization of globulins stored in the cotyledons first sets in after this time. Thus, for the early stages in germination the radicle/young root is autonomous in terms of amino acid provision. However, this situation and its timing varies considerably from species to species (Tiedemann et al., 2000).

If there is no new synthesis of storage globulins during the growth of the radicle and primary root for the first days of germination, how is it that after 3 days of germination storage globulins synthesized during embryogenesis are still distributed in the cells of the root tip, including the cells of the calyptra which are products of the root apical meristem? If storage globulin synthesis in the embryonic axis/radicle no longer occurs during germination, one would expect an increasingly weaker globulin signal in the newly formed cells of the root tip as it grows away from the seed, but this does not appear to be the case. In addition, if the globulins seen in the young root were merely being carried over out of the embryo they should be present as processed forms. That they are not, is an indication that they were newly synthesized during germination in tissues lacking processing enzymes.

The situation with barley lectin in the vacuoles of the root tip of 3 day old barley seedlings may be different to the seed storage globulins of pea seedlings. Our immunogold EM investigations confirm the presence of the barley lectin in vacuoles in the root tip of barley. This is not unexpected, since other lectins, e.g. phytohemagglutinin are known to be synthesized in the embryonic axis and root tips of bean seedlings (Greenwood et al., 1984; Kjemtrup et al., 1995), and play roles in defence against pathogens (Chrispeels and Raikhel, 1991) and in *Rhizobium* recognition (Bohlool and Schmidt, 1974). Unclear, however, is whether the lectin-positive electron opaque deposits in barley root tip vacuoles are made up exclusively of barley
lectin, or whether other storage proteins contribute to these aggregates. Nevertheless, we have screened sections in and around the meristem, in the calyptra and in the flank region of the root cortex principally in barley, but also in pea root tips, and were unable to detect a population of vacuoles which stained exclusively with a single TIP. Double immunogold labeling of sections showed that both $\alpha$ and $\gamma$ TIPs were always present together in the same tonoplast. Although our observations were concentrated on cells in the root tip, in particular in barley, we did notice that cells in the elongation zone had progressively less detectable $\alpha$-TIP. Thus, in contrast to Paris et al. (1996) who used the same plant materials, our data show that cells arising from the meristem in roots do not develop separate PSV and LV compartments which ultimately fuse to form a central vacuole. Instead, initially only a single vacuole type is formed which has with mixed PSV and LV characteristics. This may be due to the insertion of newly synthesized $\gamma$-TIP into the original PSV creating a hybrid vacuole which is then gradually transformed into a central vacuole of the LV type as elongation and differentiation proceed.

Our data qualify, but do not revoke the multi-compartment concept for the plant vacuole. While root tips of young barley and pea seedlings do not develop separate populations of PSV- and LV- type vacuoles, the literature (cited above) contains sufficient examples for different vacuole populations in a single cell. Perhaps the most convincing example are the storage parenchyma cells in the cotyledons of developing pea seeds. Here not only are PSVs from LVs easily distinguishable from one another in sections in the electron microscope, but tonoplast membrane fractions with different isopycnic densities and different TIPs can be isolated from this tissue at different developmental stages (Hoh et al., 1995). The recent paper of Otegui et al. (2006) in which the presence of only a single vacuole during the main phase of storage protein deposition in developing Arabidopsis embryos might seem to stand in contradiction to the earlier results of Hoh et al. (1995). However, a recent time-course analysis of ultrastructural changes during embryo development in Arabidopsis (Wie, Hinz, Robinson, unpublished) has revealed a series of events closely similar to those described by Hoh et al. (1995) for vacuole biogenesis in pea cotyledons, namely the de novo
development of an initially tubular PSV (not unlike that depicted in Fig. 6 D) in the early bent cotyledon stage which quickly supersedes the previous LV (Wie, Hinz and Robinson, in preparation).

MATERIALS AND METHODS

Plant materials

Barley (Hordeum vulgare L.) var. “Madonna” (Lochow-Petkus GmbH, Bergen, Germany) and pea (Pisum sativum L.) var. “Kleine Rheinländerin” (Wagner, Heidelberg, Germany) were used in this study. For 3-day old seedlings, seeds were imbibed for 30 min (barley) or 3 h (pea), placed on moist filter papers and germinated at room temperature in the dark. For 10-day old seedlings, seeds were potted in TKS2 soil (Floragard, Oldenburg, Germany), after imbibition and grown in a 12 h white fluorescent light (430W Son-T Agro bulbs, Philips, Hamburg, Germany) and 12 h dark regime, at 22-25°C at 40-60% relative humidity in a greenhouse.

Antibodies

The antisera and their dilutions used for (Western blotting) and [Immunofluorescence, immunogold EM] were: anti-α-TIP (1:1000) [1:50] against the C-terminal sequence HQPLAPEDY of Arabidopsis α-TIP (Jauh et al. 1998); anti-γ-TIP (1:1000) [1:50-400] against the C-terminal sequence CCSRTHEQLPTTDY of Arabidopsis γ-TIP (Jauh et al. 1998); anti-γ-TIP [1:50] against the C-terminal sequence INQNGHEQPTTDY of radish VM23 (Suga and Maeshima, 2004); anti-γ-TIP against the total VM23 protein isolated from radish (Maeshima, 1992); anti-δ-TIP (1:1000) 1:50 against the C-terminal sequence HVPLASADF of Arabidopsis δ-TIP (Jauh et al., 1998); anti-V-ATPase [1:50] against the A-polypeptide of the V₅ subunit of V-ATPase from Mesembryanthemum crystallinum (Haschke et al., 1989) ; anti-WGA (wheat germ agglutinin) (1:1000) [1:300-400] (Sigma, Taufkirchen, Germany); anti-legumin (1:10.000) [1:50] against Pisum sativum legumin (Hinz et al., 1999); anti-vicilin (1:1000) [1:50-100] against Vicia faba vicilin (Dr. R Manteufel, Gatersleben, Germany).

Light microscopy
Sample preparation for immunofluorescence light microscopy

3 mm root tips from were excised from pea and barley seedlings and fixed in 3.7% (w/v) formaldehyde in PIPES buffer (50 mM PIPES-KOH, pH 6.9, 5 mM MgSO₄·7H₂O, 5 mM EGTA) for 60 min at RT, washed in PIPES buffer for 30 min at room temperature, then in PBS (10 mM K₂HPO₄/KH₂PO₄ pH 7.4, 150 mM NaCl) for 30 min at RT. Samples were then dehydrated via an ethanol series: 30% (v/v) EtOH in PBS for 30 min, 50% EtOH in PBS for 30 min, 70% EtOH in PBS for 30 min, and 99% EtOH in PBS for 30 min. Samples were then stained in 0.01% (w/v) Toluidine Blue in 99% (v/v) EtOH for 5 min at RT and washed in 99% (v/v) EtOH for 5 min.

Wax embeddment followed the Steadman procedure (Steadman, 1957), essentially using a wax (90% (w/v) polyethylene glycol di-stearate (400 g x mol⁻¹, 10% (v/v) 1-hexadecanol) that after preparation melts at 37°C. Stained samples in ethanol were added to a similar volume of wax and left to infiltrate overnight at 37°C. Next day, first the ethanol overlay and then the wax around the samples was removed and new wax added and was incubated for another 2 h before being transferred to RT. Sections (10 µm) were cut with a microtome (Jung, Nussloch, Germany) equipped with a steel-knife (Leica, Bensheim, Germany) and transferred to glass slides previously coated with 10 µl glycerol-albumin. The sections were stretched with water and, after removing the water the slides were left to dry for at least 15 h. Prior to immunolabeling, the wax was removed from the sections through an ethanol series: 99% (v/v) EtOH for 4 x 10 min; 90%(v/v) EtOH in PBS for 10 min; 70% (v/v) EtOH in PBS for 10 min; 50% (v/v) EtOH in PBS for 10 min; 25% (v/v) EtOH in PBS for 10 min; and PBS for 10 min.

Immunofluorescence labeling

Sections on glass-slides were incubated at RT with 100 µl each of the following: BSA-block solution (1% (w/v) BSA in PBS) for 5 min; glycine-block solution (20 mM glycine in PBS) for 5 min; primary antibody diluted in wash buffer (0.1% (w/v) BSA in PBS) for 1 h; wash buffer for 3 x 10 min; secondary antibody (Alexa Fluor 488 goat-anti-rabbit IgG (H+L) 2 mg.ml⁻¹ (Molecular Probes, Leiden, Netherlands) 1:50 with wash buffer for 1 h; wash buffer for 3 x 5 min, then PBS for 3 x 5 min.. A covering solution was prepared by dissolving 100 mg p-phenylenediamine in 10 ml PBS, then 90 ml glycerol was added and the solution was brought to pH 8.0 with Tris. A drop of the cover solution was added to the labelled sections before they were mounted and sealed with nail varnish.
Examination of the immunofluorescence labelling followed in an Axiovert 200 microscope (Zeiss, Göttingen, Germany) using the FT 510 filter (Zeiss, Göttingen, Germany) emitting light at 450-490 nm and 515-565 nm. Images were captured with a Canon G2 digital camera.

**Immunogold Electron Microscopy (IEM)**

**Sample preparation**

Pea and barley root tips as above were fixed in [1.5% paraformaldehyde, 0.25% glutaraldehyde in wash buffer (0.1 M K$_2$HPO$_4$/KH$_2$PO$_4$ pH 7.0)] first for 30 min at RT then overnight at 4°C. Samples were dehydrated through an ethanol series [30% (v/v) EtOH in H$_2$O for 30 at 4°C; 50% (v/v) EtOH for 15 at 4°C then for 45 min at -20°C; 70% (v/v) for 60 min at -20°C; 100% (v/v) for 2 x 60 min at -20°C] using the PLT (progressive lowering of temperature) technique in an AFS freeze substitution unit (Leica, Bensheim, Germany). Samples were then embedded in HM20 (Lowicryl HM Kit, Polyscience, Eppelheim, Germany) [25% (v/v) HM20 in EtOH for 45 min at -20°C; 50% (v/v) HM20 in EtOH for 45 min at -20°C; 75% (v/v) HM20 in EtOH for 45 min at -20°C; 100% (v/v) HM20 for 1.5h at -35°C; 100% (v/v) HM20 for 4 h at -35°C], and polymerized in UV-light at -35°C. 90 nm thick sections were cut with an Ultracut S microtome (Reichert, Vienna, Austria), and transferred to Formvar-coated nickel-grids.

**Single antibody labeling**

HM20-embedded sections on grids were blocked with 3% (w/v) BSA in PBS (10 mM K$_2$HPO$_4$/KH$_2$PO$_4$ pH 7.4, 150 mM NaCl) for 30 min, incubated in primary antibody for 1 h, washed in washing buffer (1% (w/v) BSA in PBS) for 3 x 10 min, incubated with secondary antibody [gold-conjugated goat-anti-rabbit IgG with 5, 10 or 15 nm gold particles (EM.GAR 5, 10 or15; British BioCell International, Cardiff, GB)], diluted 1:50 with PBS for 1 h, washed in washing buffer for 2 x 5 min and in H$_2$O for 3 x 5 min.

**Double antibody labeling**

Since all the primary antisera were generated in rabbits, a double labeling procedure was performed by first gently placing an HM20-embedded section, directly after cutting, onto a drop of blocking solution. Most of the drop was removed afterwards with a fine syringe, and the next solution added by carefully under-layering it, still allowing the section to float. After the incubation and washing regime (described below), the section was gently transferred to a Formvar-coated grid by sliding the grid...
under the section so that the upper (non-labeled) side of the section was now accessible for a new round of labeling with another primary antibody (also as below). Labeling was done using similar solutions as above: Blocking in block buffer for 1 x 2 min, then for 1 x 30 min, primary antibody incubation for 1h, washing in wash buffer 1 x 2 min, then for 3 x 10 min, secondary antibody incubation for 1 h, washing in wash buffer for 3 x 5 min and in H2O for 3 x 5 min. The sections were post-stained in 3% (w/v) uranyl-acetate in H2O for 5 min, washed in H2O for 3 x 1 min, then incubated in 0.3% (w/v) lead-citrate in 1 M NaOH for 5 min, washed in H2O for 4 x 1 min. After drying, samples were examined in a Philips CM10 transmission electron microscope.

**Cryosectioning**

Cryosections were prepared, and labeled with primary and secondary antibodies exactly as described in Pimpl et al. (2000).

**Protein extraction, SDS-PAGE and Western blotting**

*Total membrane protein fractions from barley and pea roots.*

3 mm root tips were cut and homogenized with acid-washed sea sand in 10-fold volume of homogenisation buffer (40 mM HEPES-KOH pH 7.0, 300 mM sucrose, 10 mM KCl, 3 mM MgCl2) including protease inhibitors (1 mM DTT, 2 µg.ml⁻¹ aprotinin, 0.5 µg.ml⁻¹ leupeptin, 2 µg.ml⁻¹ pepstatin, 2 mM o-phenanthroline, 1 µg.ml⁻¹ E-64). The homogenate was passed through one layer of Miracloth and centrifuged at 1000 xg in a Sorvall HB 4 rotor for 10 min. The pellet was discarded and the supernatant was centrifuged at 12.000g for 20 min in the same rotor. The pellet was saved and the supernatant centrifuged at 100.000g for 1h in a Sorvall TFT 50.38 rotor. The supernatant was discarded and the pellets were resuspended in homogenisation buffer using a glass homogenizer. Protein concentrations were measured according to Lowry (Lowry et al., 1951) and protein samples for SDS-PAGE were precipitated with methanol/chloroform (Wessel and Flügge, 1984).

*Isolation of protein bodies from pea cotyledons.*

Pea seeds were harvested 4 weeks after flowering, the testa was removed and the seeds weighed. The isolation procedure was according to Hohl et al. (1996) and Hinz et al. (1999). Essentially, two volumes of homogenisation buffer (0.1 M MOPS-KOH pH 5.5, 0.6 M Sorbitol, 1 mM EDTA) with antiproteases as above were added and the plant material was finely chopped by hand with a razor blade. The homogenate was filtered through 1x Miracloth and centrifuged at 90xg in a Labofuge I swing-out
rotor (Labofuge, Hereaus-Christ, Osterode am Harz, Germany) for one min. The supernatant was loaded onto a 5% (w/v) Ficoll 400 cushion and centrifuged at 460xg in a Sorvall HB4 rotor for 10 min. The pellet (material collected from the top of the cushion) was resuspended in homogenisation buffer and re-centrifuged, this step was then repeated once more. The pellet was saved; protein concentration determination and sample preparation for SDS-PAGE was done as described above.

Isolation of integral membrane proteins from barley leaves.
Leaves from 5-days old barley seedlings were homogenized with acid-washed sea-sand in (40 mM HEPES/KOH pH 7.0, 300 mM sucrose, 10 mM KCl, 3 mM MgCl) with antiproteases as above. The homogenate was passed through 1x Miracloth and centrifuged at 18,000xg in a Sorvall HB4 rotor for 20 min. The pellet was discarded and the supernatant was centrifuged at 100,000xg in a Sorvall TFT 50.38 rotor for 1 h. The pellet was resuspended in 2 ml homogenisation buffer and diluted 1:10 with potassium-iodine solution (1M KI in 20 mM MES-KOH pH 7.0), incubated under rotation for 30 min. at 4°C to remove peripheral membrane proteins. The solution was then centrifuged again at 100,000xg for 1 h, and the pellet was resuspended in 20 mM MES-KOH pH 7.0, and prepared for SDS-PAGE as described above.

Western blotting.
After electrophoretic separation on 10-20% polyacrylamide gradient gels, using standard procedures, proteins were transferred to Nitrocellulose membranes (Pall, Pensacola, FL, USA), blocked in 5% (w/v) milk powder in TBST (50 mM Tris-HCl pH 7.5, 200 mM NaCl, 0.05% (v/v) Tween 20) and incubated with antisera diluted in TBS with 1% (w/v) BSA. Washing procedures, secondary HRP-coupled antibody incubation and detection of luminescence signals followed essentially the instructions of the “SuperSignal West Pico Chemiluminescence Kit” (PIERCE, Bonn, Germany).

ACKNOWLEDGEMENTS

Financial support of the German Research Council (DFG Ro 440/13-3) is gratefully acknowledged. AO thanks the Landesgraduiertenförderung of Baden-Württemberg for a stipend.
LITERATURE CITED

Amelunxen F, Heinze U (1984) Zur Entwicklung der Vakuole in Testa-Zellen des Leinsamens. Eur J Cell Biol 35: 343-354
Barrieu F, Thomas D, Marty-Mazars D, Marty F (1998) Tonoplast intrinsic protein from caulilower (Brassica oleracea L. var. botrytis): Immunological analysis, cDNA cloning and evidence for expression in meristematic tissues. Planta 204: 335-344
Bewley JD, Black M (1994) Seeds. Physiology of development and germination. 2nd edition. Plenum Press, New York.
Bohlool BB, Schmidt E (1974) Lectins- possible basis for specificity in rhizobium-legume root nodule symbiosis. Science 185: 269-271
Chandrasekharan MB, Bishop KJ, Hall TC (2003) Module-specific regulation of the ß-phaseolin promoter during embryogenesis. Plant J 33: 853-866
Chrispeels MJ, Raikhel NV (1991) Lectins, lectin genes, and thes role in plant defense. Plant Cell 3: 1-9
Chrispeels MJ, Maurel C (1994) Aquaporins - the molecular-basis of facilitated water-movement through living plant-cells. Plant Physiol 105: 9-13
Chrispeels MJ, Daniels MJ, Weig A (1997) Aquaporins and water transport across the tonoplast. Advances in Botanical Research 25: 419-432
De DN (2000) Plant Cell Vacuoles. An Introduction. CSIRO Publishing, Collingwood, Australia
Di Sansebastiano GP, Paris N, Marc-Martin S, Neuhaus JM (1998) Specific accumulation of GFP in a non-acidic vacuolar compartment via a C-terminal propeptide-mediated sorting pathway. Plant J 15: 449-457
Di Sansebastiano GP, Paris N, Marc-Martin S, Neuhaus J-M (2001) Regeneration of a lytic central vacuole and of neutral peripherla vacuoles can be visualized by green fluorescent proteins targeted to either type of vacuole. Plant Physiol 126: 78-86
Diwu ZJ, Chen CS, Zhang CL, Klaubert DH, Haugland RP (1999) A novel acidotrophic pH indicator and its potential application in labelling acidic organelles in live cells. Chemistry and Biology 6: 411-418
Epimashko S, Meckel T, Fischer-Schliebs E, Lüttge U, Thiel G (2004) Two functionally different vacuoles for static and dynamic purposes in one plant mesophyll leaf cell. Plant J 37: 294-300
Fleurat-Lessard P (1988) Structural and ultrastructural features of cortical cells in motor organs of sensitive plants. Biol Rev 63: 1-22
Fleurat-Lessard P, Frangne N, Maeshima M, Ratajczak R, Bonnemain JL, Martinoia E (1997) Increased expression of vacuolar aquaporin and H-ATPase related to motor cell function in *Mimosa pudica* L. Plant Physiol 114: 827-834

Fleurat-Lessard P, Michonneau P, Maeshima M, Drevon JJ, Serraj R (2005) The distribution of aquaporin subtypes (PIP1, PIP2 and gamma TIP) is tissue dependent in soybean (*Glycine max*) root nodules. Ann Bot (London) 96: 457-460.

Flückinger R, De Caroli M, Piro G, Dalessandro G, J-M Neuhaus Di Sansebastiano GP (2003) Vacuolar system distribution in Arabidopsis tissues, visualized using GFP fusion proteins. J Exp Bot 54: 1577-1584

Franceschi VR, Wittenbach VA, Giaquinta RT (1983) Paraveinal mesophyll of soybean leaves in relation to assimilate transfer and compartmentation: III Immunohistochemical localization of specific glycoproteins in the vacuole after depodding. Plant Physiol 72: 586-589

Greenwood JS, Keller GA, Chrispeels MJ (1984) Localization of phytohemagglutinin in the embryonic axis of *Phaseolus vulgaris* with ultra-thin cryosections embedded in plastic after indirect immunolabeling. Planta 162: 548-555

Hall TC, Chandrasekharan MB, Li G (1999) Phaseolin: its past, properties, regulation, and future. In: Shewry PR, Casey R, eds. Seed Proteins. Dordrecht, The Netherlands: Kluwer Academic Publishers, pp. 209-240

Hara-Nishimura I, Maeshima M (2000) Vacuolar processing enzymes and aquaporins. In DG Robinson and JC Rogers, eds, Vacuolar Compartments, Annual Plant Reviews Vol. 5. Sheffield Academic Press, Sheffield, pp. 20-42

Haschke HP, Bremberger C, Lüttge U (1989) Transport proteins in plants with crassulacean metabolism: immunological characterization of ATPase subunits. In: Plant Membrane Transport: The Current Position. J. Dainty, M.I. DeMichaelis, E. Marré, E. Rasi-Caldogno, eds. (Elsevier, Amsterdam) pp. 149-154

Hinz G, Hillmer S, Bäumer M, Hohl I (1999) Vacuolar storage proteins and the putative vacuolar sorting receptor BP-80 exit the Golgi apparatus of developing pea cotyledons in different transport vesicles. Plant Cell 11: 1509-1524

Hinz G, Colanesi S, Hillmer S, Rogers JC, Robinson DG (2007) Localization of vacuolar transport receptors and cargo proteins in the Golgi apparatus of developing Arabidopsis embryos. Traffic doi:10.1111/j.1600-0854.2007.00625.x

Höfte H, Hubbard L, Reizer J, Ludevid D, Herman EM, Chrispeels MJ (1992) Vegetative and seed-specific forms of tonoplast intrinsic protein in the vacuolar membrane of *Arabidopsis thaliana*. Plant Physiol 99: 561-570

Hoh I, Hinz G, Jeong B-K, Robinson DG (1995) Protein storage vacuoles form de novo during pea cotyledon development. J Cell Sci 108: 299-310
Jauh GY, Fischer AM, Grimes HD, Ryan CA, Rogers JC (1998) Delta-tonoplast intrinsic protein defines unique plant vacuole functions. PNAS USA 95: 12995-12999

Jauh G-Y, Phillips T, Rogers JC (1999) Tonoplast intrinsic protein isoforms as markers for vacuole functions. Plant Cell 11: 1867-1882

Jiang L, Phillips TE, Rogers SW, Rogers JC (2000) Biogenesis of the protein storage crystalloid. J Cell Biol 150: 755-770

Jiang L, Phillips TE, Hamm CA, Drozdowicz YM, Rea PA, Maeshima M, Rogers SW, Rogers JC (2001) The protein storage vacuole: a unique compound organelle. J Cell Biol 155: 991-1002

Johnson KD, Höfte H, Chrispeels MJ (1989) An abundant, highly conserved tonoplast protein in seeds. Plant Physiol 91: 1006-1013

Kjemtrup S, Borksenious O, Raikhel NV, Chrispeels MJ (1995) Targeting and release of phytohemagglutinin from the roots of bean seedlings. Plant Physiol 109: 603-610

Lowry OH, Rosenbrough NJ, Farr LA, Randell RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193: 265-275

Ludevid D, Höfte H, Himmelblau E, Chrispeels MJ (1992) The expression pattern of the tonoplast intrinsic protein gamma-TIP in Arabidopsis thaliana is correlated with cell enlargement. Plant Physiol 100: 1633-1639

Maeshima M (1992) Characterization of the major integral protein of vacuolar membrane. Plant Physiol 98: 1248-1254

Marty F (1999) Plant vacuoles. Plant Cell 11: 587-600

Moriyasu Y, Hattori M, Jauh GY, Rogers JC (2003) Alpha tonoplast intrinsic protein is specifically associated with vacuole membrane involved in an autophagic process. Plant Cell Physiol 44: 795-802

Müntz K, Shutov, AD (2002) Legumains and their functions in plants. Trends in Plant Science 7: 340-344

Otegui MS, Herder R, Schulze J, Jung R, Staehelin LA (2006) The proteolytic processing of seed storage proteins in Arabidopsis cells starts in the multivesicular bodies. Plant Cell 18: 2567-2581

Neuhaus JM, Paris N (2006) Plant Vacuoles: from biogenesis to function. In Plant Endocytosis, 1. J. Samaj, F. Baluska, and D. Menzel, eds. (Berlin Heidelberg: Springer-Verlag) p.63-82

Paris N, Stanley CM, Jones RL, Rogers JC (1996) Plant cells contain two functionally distinct vacuoles. Cell 85: 563-572
Park M, Kim SJ, Vitale A, Hwang I (2004) Identification of the protein storage vacuole and protein targeting to the vacuole in leaf cells of three plant species. Plant Physiol 134: 625-639

Pernollet JC (1978) Protein bodies of seeds: biochemistry, biosynthesis, and degradation. Phytochemistry 17:1473-1480

Pimpl P, Movafeghi A, Coughlan S, Denecke J, Hillmer S, Robinson DG (2000) In situ localization and in vitro induction of plant COPI-coated vesicles. Plant Cell 12: 2219-2235

Preston GM, Carroll TP, Guggino WB, Agre P (1992) Appearance of water channels in Xenopus oocytes expressing red-cell CHIP28 protein. Science 256: 385-387

Robinson DG, Hinz G (1997) Vacuole biogenesis and protein transport to the plant vacuole: a comparison with the yeast vacuole and animal lysosome. Protoplasma 197: 1-25

Robinson DG, Rogers JC (2000) Vacuolar Compartments. Ann. Plant Reviews Vol. 5, Sheffield Academic Press, Sheffield, UK

Sanmartin M, Ordonez A, Sohn EJ, Robert S, Sanchez-Serrano JJ, Surpin MA, Raikhel NV, Rojo E (2007) Divergent functions of VTI12 and VTI11 in trafficking to storage and lytic vacuoles in Arabidopsis. PNAS USA 104: 3645-3650

Serraj R, Fragne N, Maeshima M, Fleurat-Lessard P, Drevon JJ (1998) A gamma-TIP cross-reacting protein is abundant in the cortex of soybean N2-fixing nodules. Planta 206: 681-684

Staswick PE (1994) Storage proteins of vegetative plant tissue. Annu Rev Plant Physiol Plant Mol Biol 45: 303-322

Steadman, HF (1957) Polyester Wax: A New Ribboning Embedding Medium for Histology. Nature 179: 1345

Swanson SJ, Bethke PC, Jones RL (1998) Barley aleurone cells contain two types of vacuoles. Characterization of lytic organelles by use of fluorescent probes. Plant Cell 10: 685-698

Suga S, Maeshima M (2004) Water channel activity of radish plasma membrane aquaporins heterologously expressed in yeast and their modification by site-directed mutagenesis. Plant Cell Physiol. 45: 823-830

Tiedemann J, Neubohn B, Müntz K (2000) Different functions of vicilin and legumin are reflected in the histopattern of globulin mobilization during germination of vetch (Vicia sativa L.) Planta 211: 1-12
Tomos AD, Leigh RA, Koroleva OA (2000) Spatial and temporal variation in vacuolar contents. In DG Robinson and JC Rogers, eds, Vacuolar Compartments, Annual Plant Reviews Vol 5. Sheffield Academic Press, Sheffield, pp174-198

Vitale A, Hinz G (2005) Sorting of proteins to storage vacuoles: how many mechanisms? TIPS 10: 316-323

Wessel D, Flügge UI (1984) A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Anal Biochem 138: 141-143

Wink M (1993) The plant vacuole - A multifunctional compartment. J Exp Bot 44: 231-246 Suppl.

Zakharov A, Giersberg M, Hosein F, Melzer M, Müntz K, Saalbach I (2004) Seed-specific promotors direct gene expression in non-seed tissue. J Exp Bot 55: 1463-1471

LEGENDS TO FIGURES

Figure 1. Immunological detection of TIPs. A) $\alpha$-TIP, but not $\delta$-TIP is present in membranes extracted from 1 mm segments excised from root tips of 3 day old barley seedlings. As controls for PSV membranes a protein body fraction was isolated from pea seeds. Two types of $\alpha$-TIP antisera were used: against $\alpha$-TIP – total protein (Johnson et al., 1989), and against a C-terminal peptide (Jauh et al., 1999). B) $\gamma$-TIP is also present in membranes from barley root tips. Again, two different antisera ($\gamma$-TIP antibodies generated against a C-terminal peptide, Jauh et al., 1998; $\gamma$-TIP (= VM23) total protein antibodies, Maeshima et al., 1992) gave the same result (data not shown). A membrane fraction isolated from barley leaves is used as a control for LV membranes. C) Barley lectin (= wheat germ agglutinin, WGA) is present in root tips but not leaves of barley. D) Detection of TIPs and unprocessed forms of storage globulins in 1 mm root tip segments from 3 day old pea seedlings. Note that legumin, when processed has two polypeptides at 40 and 20 kDa (linked by a disulfide bridge). Our antiserum (Hinz et al., 1999) recognizes only the 60 kDa precursor and the 40 kDa mature polypeptide, which are shown in the blot, but not the 20 kDa polypeptide. E) Controls for immunogold detection of TIPs; pea cotyledon protein bodies for $\alpha$– and $\delta$-TIPs; vacuoles in elongation zone of barley roots for $\gamma$-TIP. Cryosections labelled with peptide-antisera (Jauh et al., 1998). Bars = 100 nm.

Figure 2. Localization of barley lectin and $\alpha$-TIP in root tips of 3-day old barley seedlings. A) A phase contrast picture of longitudinal section. B – D) Lectin is found
in the cells of the calyptra, the rhizodermis and the outermost cells of the cortex. Inset: high magnification of a single calyptra cell. The lectin signal is more intense at the rims of the vacuoles, as seen in EM sections (Fig. 6). E – H) α-TIP has a similar distribution to barley lectin, except that it is absent from the innermost cells of the calyptra. E, H) α–TIP total protein antiserum (Johnson et al., 1989) G) α-TIP peptide antiserum (Jauh et al., 1998). Arrowhead points to the meristem. Bars = 100 μm

Figure 3. Localization of γ-TIP in root tips of 3-day old barley seedlings with γ-TIP peptide antiserum (Suga and Maeshima, 2004). The distribution of γ-TIP is similar to that of barley lectin, and is restricted to the calyptra, rhizodermis and outermost cortical cells. A) longitudinal section, B) transverse section above the meristem, C) transverse section through the calyptra. Bars = 50 μm.

Figure 4. Localization of vicilin and α-TIP in root tips of 3-day old pea seedlings. A) Phase contrast picture of longitudinal section. B) Vicilin is present only at the outermost cells of the calyptra, but also in the innermost cells of the cortex. Inset: high magnification of a single cell at the flank of the meristem revealing peripheral vicilin deposits in vacuoles. C – E) α-TIP is expressed by nearly all cells of the root tip except columella cells. α-TIP was detected by α-TIP total protein antiserum (Johnson et al., 1989); the same results were obtained α-TIP peptide antibodies (Jauh et al., 1998) – data not shown. Bars = 100 μm (A,B), 50 μm (C-E).

Figure 5. Localization of γ-TIP in root tips of 3-day old pea seedlings. γ-TIP is strongly expressed by the innermost cells of the cortex, but is lacking from the central cylinder and rhizodermis. The root cap shows a γ-TIP signal that is more pronounced in the outer cell layers. A) – longitudinal section. Inset: high magnification of a single cell from the cortex shows γ-TIP labeling is restricted to the periphery of vacuoles. B, C) transverse sections in regions above (B) and in (C) the region of the meristem. Bars = 100 μm.

Figure 6. Immunogold detection of barley lectin in vacuoles of 3 day-old barley roots. A) A cell in the calyptra revealing electron opaque aggregates within the vacuoles. B-D) The electron opaque aggregates stain positively with anti wheat germ agglutinin gold (10 nm) conjugates. C, D) Stages in vacuole formation in cells immediately bordering on the meristem. Barley lectin-positive aggregates are positioned more on
the outer than the inner tonoplast membrane. Bars = 1.5 µm (A), 400 nm (B), 150 nm (C), 300 nm (D).

**Figure 7.** Immunogold detection of TIPs in cells from 3-day old barley roots. A, C) Double immunogold labeling with α-TIP (15 nm gold particles, arrowheads; α-TIP peptide antiserum, Jauh et al., 1998), and γ-TIP (5 nm gold, arrows; γ-TIP peptide antiserum, Jauh et al., 1998). B) Single immunogold labeling of a PSV (note storage protein aggregates on the tonoplast) with γ-TIP peptide antiserum (10 nm gold, Jauh et al., 1998). D) Longitudinal section of root for cell positioning. Boxes indicate areas in the cortex and calyptra from which the vacuoles in (A) and (C) are depicted. Bars = 100 nm (A, C), 200 nm (B).

**Figure 8.** Immunogold detection of vicilin and TIPs in 3-day old root tips of pea seedlings. A) Vicilin-positive immunogold labeling (10 nm gold particles) of a vacuole from the inner cortex; cryosection, B) Double immunogold labeling with α-TIP (15 nm gold, arrowheads; α-TIP peptide antiserum. Jauh et al., 1998), and γ-TIP (5 nm gold, arrows, γ-TIP peptide antiserum, Jauh et al., 1998) of a PSV from the same cell type. Bars = 150 nm (A), 200 nm (B).

**Supplementary Figures**

**Figure 1.** Immunological detection of storage proteins (barley lectin, vicilin) and TIPs in root tips of 10 day old barley (A-C) and pea (D-F) seedlings. A) barley lectin; B, E) α-TIP labeled with α-TIP peptide antiserum, Jauh et al., 1998; C, F) γ-TIP labeled with γ-TIP peptide antiserum, Suga and Maeshima, 2004) Bars = 100 µm.

**Figure 2.** Immunological detection of V-ATPase in root tips of 3 day old barley (A) and pea (B) seedlings. Bars = 100 µm.
